

TAMOXIFEN METABOLITES CAN TARGET BOTH AROMATASE AND  
ESTROGEN RECEPTORS

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## **DEDICATION**

This work is dedicated to my grandfather Yongzeng, who showed me the greatness of perseverance, and who no longer has the chance to read the story of my dissertation.

This work is dedicated to my father Yunbo, who granted me the capability to listen and learn, and who gave up his dreams to support mine.

This work is dedicated to my grandmother Yongxiang, who shared the joy and innocence of her life with me.

This work is dedicated to my wife Cong, who has traveled this journey with me and who has sacrificed so much for the future of our family.

This work is dedicated to my aunts Yunxia and Yunhua, and my uncle Yunwei, who have dedicated so much for my personal growth.

This work is dedicated to my cousins Qinshu, Junna and Da, who I hope will one day find this story of my Ph.D. training bringing passion to their own lives.

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## TAMOXIFEN METABOLITES CAN TARGET BOTH AROMATASE AND ESTROGEN RECEPTORS

Breast cancer remains the most prevalent malignancy diagnosed in women. More than two thirds of all diagnosed breast cancers are estrogen receptor (ER)-positive and are dependent on estrogen signaling. Drugs for the treatment of ER-positive breast cancer can be divided into three classes: selective estrogen receptor modulators (SERMs), selective estrogen receptor down-regulators (SERDs) and aromatase inhibitors (AIs). However, the efficacy and safety of SERMs, SERDs and AIs are compromised by side effects or tumor resistance. One possible way of improving treatment efficacy and safety profiles is to develop agents with dual aromatase inhibitory and ER modulatory activity.

Over the past 30 years, tamoxifen, a SERM, has become the most widely used drug for the adjuvant treatment of breast cancer. The metabolism of tamoxifen has a complex profile involving both active and inactive metabolites, among which endoxifen, 4-hydroxytamoxifen (4-HT) and norendoxifen (Nor) have been shown to have ER modulatory activity. Previous studies have also shown that norendoxifen is a potent AI *in vitro*. These preliminary studies support the utilization of tamoxifen metabolites as lead compounds for the development of dual AI/SERM(D) agents.

Hydroxynorendoxifen (Hdn) was identified as a novel tamoxifen metabolite, with an average plasma concentration of 0.82 nM. Nor and Hdn were potent and relatively selective AIs, with  $K_i$ s of 70 nM and 20 nM, respectively. Nor and Hdn have high binding affinity for ER- $\alpha$  and ER- $\beta$ , with  $EC_{50}$  values less than 35 nM. Nor and Hdn can inhibit breast cancer cell proliferation with high potency, with  $IG_{50}$ s of 25 nM and 9 nM, respectively. Nor and Hdn can suppress progesterone receptor (PGR) mRNA expression level by reducing it by 68% and 86%. Moreover, a series of Nor analogues were shown to have both potent aromatase inhibitory activity and high ERs binding affinity.

Results from this dissertation will contribute to three aspects: 1) the identification of Hdn as a tamoxifen metabolite illustrated a more comprehensive metabolism profile of tamoxifen; 2) the data suggest Nor and Hdn possess dual aromatase inhibitory and ER antagonistic activity; 3) a series of Nor analogues were characterized as lead compounds for the development of dual AI/SERM(D) agents.

David A. Flockhart, M.D., Ph.D., Chair

## TABLE OF CONTENTS

|   |       |
|---|-------|
| LIST OF TABLES .....  | xviii |
| LIST OF FIGURES .....   | xx    |
| LIST OF ABBREVIATIONS .....   | xxii  |
| CHAPTER 1: INTRODUCTION .....   | 1     |
| 1.1 Breast cancer and its classification .....  | 1     |
| 1.2 Breast cancer management and treatment .....  | 3     |
| 1.3 Estrogen receptor-positive cancer and estrogen signaling .....                              | 5     |
| 1.4 Selective estrogen receptor modulators (SERMs).....   | 8     |
| 1.4.1 Triphenylethylene derivatives.....  | 9     |
| 1.4.2 Benzothiophene derivatives .....  | 11    |
| 1.5 Selective estrogen receptor down-regulators (SERDs) .....                                   | 11    |
| 1.5.1 Fulvestrant .....   | 12    |
| 1.6 Aromatase and aromatase inhibitors .....  | 12    |
| 1.6.1 Aromatase.....  | 12    |
| 1.6.2 Aromatase inhibitors .....  | 14    |
| 1.7 Tamoxifen and its metabolites .....   | 16    |
| 1.8 Comparison of SERMs, SERDs and AIs for the treatment of ER-<br>positive breast cancer ..... | 20    |
| 1.8.1 Tamoxifen versus AIs .....  | 20    |

|   |    |
|---|----|
| 1.8.2 Tamoxifen versus fulvestrant .....  | 22 |
| 1.8.3 Fulvestrant versus Als .....  | 22 |
| 1.9 Side effects of SERMs, SERDs and Als for the treatment of ER-<br>positive breast cancer .....     | 23 |
| 1.9.1 Side effects of tamoxifen.....  | 23 |
| 1.9.2 Side effects of fulvestrant.....  | 24 |
| 1.9.3 Side effects of Als .....   | 25 |
| 1.10 Tumor resistance to SERMs, SERDs and Als for the treatment of<br>ER-positive breast cancer ..... | 26 |
| 1.10.1 Tumor resistance to tamoxifen.....   | 26 |
| 1.10.2 Tumor resistance to fulvestrant.....   | 29 |
| 1.10.3 Tumor resistance to Als .....  | 29 |
| 1.11 Combination of SERMs/SERDs and Als for the treatment of ER-<br>positive breast cancer .....      | 31 |
| 1.11.1 Increasing treatment efficacy .....  | 31 |
| 1.11.2 Delaying or halting tumor resistance.....  | 33 |
| 1.11.3 Alleviating side effects .....   | 33 |
| 1.12 Research objectives .....  | 35 |
| CHAPTER 2: MATERIALS AND METHODS.....   | 37 |
| 2.1 Chemical and reagents .....   | 37 |

|   |    |
|---|----|
| 2.2 Breast cancer cell line .....   | 38 |
| 2.3 High-performance liquid chromatography-tandem mass<br>spectrometry (HPLC-MS/MS).....      | 38 |
| 2.3.1 Stock solutions.....  | 38 |
| 2.3.2 Instrumentation and chromatographic conditions, HPLC-MS/MS<br>operation conditions..... | 39 |
| 2.3.3 Standard curve.....   | 41 |
| 2.3.4 Assay accuracy and precision.....   | 41 |
| 2.3.5 Extraction efficiency .....   | 42 |
| 2.3.6 Extraction procedure for calibration standards.....                                     | 42 |
| 2.3.7 Analysis of patient plasma samples .....  | 43 |
| 2.4 Aromatase inhibition assay.....   | 43 |
| 2.4.1 Inhibition of recombinant human CYP isoforms by microsomal<br>incubations.....          | 43 |
| 2.4.2 Kinetic analysis of recombinant human CYP isoforms .....                                | 45 |
| 2.5 Estrogen receptor binding assay .....   | 46 |
| 2.6 Tritiated water assay .....   | 47 |
| 2.7 Gene expression analysis using real-time polymerase chain reaction<br>(PCR) .....         | 48 |
| 2.7.1 Ribonucleic acid (RNA) extraction and concentration<br>measurement.....                 | 48 |

|   |    |
|---|----|
| 2.7.2 Complementary deoxyribonucleic acid (cDNA) synthesis .....  | 49 |
| 2.7.3 Real-time quantitative polymerase chain reaction (PCR) for<br>cDNA .....  | 49 |
| 2.8 Cell proliferation assay .....  | 50 |
| 2.9 Subjects .....  | 51 |
| 2.10 Trial Design .....   | 52 |
| CHAPTER 3: AROMATASE INHIBITORY ACTIVITY AND ESTROGEN<br>RECEPTOR BINDING AFFINITY OF TAMOXIFEN METABOLITES <i>IN</i><br><i>VITRO</i> ..... |    |
| 3.1 Introduction.....   | 53 |
| 3.2 Results .....   | 55 |
| 3.2.1 Hydroxynorendoxifen as a novel tamoxifen metabolite in patients<br>taking tamoxifen.....  | 55 |
| 3.2.1.1 Chromatography .....  | 55 |
| 3.2.1.2 Linearity .....   | 57 |
| 3.2.1.3 Extraction efficiency.....  | 57 |
| 3.2.1.4 Assay accuracy and precision .....  | 58 |
| 3.2.1.5 Limit of blank (LOB), limit of detection (LOD) and lower limit<br>of quantification (LLOQ).....                                     | 59 |
| 3.2.1.6 Stability of hydroxynorendoxifen in methanol.....   | 61 |

|   |    |
|---|----|
| 3.2.1.7 Pharmacokinetic study (plasma concentration of hydroxynorendoxifen) .....   | 61 |
| 3.2.2 Potency and selectivity of aromatase inhibition by norendoxifen and hydroxynorendoxifen <i>in vitro</i> .....                             | 63 |
| 3.2.3 ER binding affinity of norendoxifen ( <i>E,Z</i> ) and hydroxynorendoxifen ( <i>E,Z</i> ) <i>in vitro</i> .....                           | 75 |
| 3.3 Discussion .....  | 76 |
| CHAPTER 4: CELLULAR AROMATASE INHIBITORY ACTIVITY AND ESTROGEN RECEPTOR ANTAGONISTIC ABILITY OF NORENDOKIFEN AND HYDROXYNORENDOKIFEN.....       |    |
|   | 84 |
| 4.1 Introduction.....   | 84 |
| 4.2 Results .....   | 85 |
| 4.2.1 Cellular aromatase activity inhibition by norendoxifen and hydroxynorendoxifen.....   | 85 |
| 4.2.2 Activity of norendoxifen and hydroxynorendoxifen to antagonize ER-responsive gene expression (in E2-stimulated breast cancer cells) ..... | 86 |
| 4.2.3 Anti-proliferation activity of norendoxifen and hydroxynorendoxifen against breast cancer cells (stimulated by estradiol) .....           | 87 |
| 4.3 Discussion .....  | 89 |



|  |     |
|--|-----|
| CHAPTER 5: CHARACTERIZATION OF NORENDOKIFEN ANALOGUES<br>WITH DUAL AROMATASE INHIBITORY ABILITY AND ESTROGEN<br>RECEPTOR ANTAGONISITIC ACTIVITY..... | 93  |
| 5.1 Introduction.....  | 93  |
| 5.2 Results .....  | 96  |
| 5.2.1 Potency of aromatase inhibition and ER binding affinity by<br>norendoxifen analogues <i>in vitro</i> .....                                     | 96  |
| 5.2.2 ER antagonistic activity of norendoxifen analogues <i>in vitro</i> .....   | 99  |
| 5.3 Discussion .....   | 102 |
| CHAPTER 6: SUMMARY AND CONCLUSIONS .....   | 105 |
| APPENDIX (Republication permission) .....  | 106 |
| REFERENCES .....   | 107 |
| CURRICULUM VITAE   |     |

## LIST OF TABLES

|   |    |
|---|----|
| Table 1. Plasma concentrations of tamoxifen, <i>N</i> -DMT, 4-HT and norendoxifen .....   | 18 |
| Table 2. Mass spectrometry settings for hydroxynorendoxifen (Hdn) and the internal standard, diphenylhydantoin (DPH) .....  | 40 |
| Table 3. Extraction efficiency (%) of hydroxynorendoxifen in human plasma .....   | 58 |
| Table 4. Intra-day and inter-day accuracy and precision (coefficient of variations, CVs) of hydroxynorendoxifen .....   | 59 |
| Table 5. Plasma concentrations of hydroxynorendoxifen in 10 women after oral administration of 20 mg tamoxifen per day for 4 months .....                                       | 61 |
| Table 6. IC <sub>50</sub> and <i>K<sub>i</sub></i> values of important tamoxifen metabolites against aromatase (CYP19) .....  | 64 |
| Table 7. IC <sub>50</sub> and <i>K<sub>i</sub></i> values of norendoxifen ( <i>E,Z</i> ) and hydroxynorendoxifen ( <i>E,Z</i> ) against important cytochrome P450 enzymes ..... | 71 |
| Table 8. IC <sub>50</sub> and <i>K<sub>i</sub></i> values of <i>E</i> - and <i>Z</i> -norendoxifen against important cytochrome P450 enzymes .....                              | 74 |
| Table 9. EC <sub>50</sub> values of important tamoxifen metabolites to ERs (ER- $\alpha$ and ER- $\beta$ ) .....  | 75 |
| Table 10. Average plasma concentrations of tamoxifen metabolites divided by corresponding <i>K<sub>i</sub></i> values against aromatase (CYP19) .....                           | 81 |
| Table 11. IC <sub>50</sub> values of norendoxifen ( <i>E,Z</i> ) and hydroxynorendoxifen ( <i>E,Z</i> ) against cellular and recombinant aromatase activity .....               | 90 |

|  |     |
|--|-----|
| Table 12. IC <sub>50</sub> values of lead norendoxifen analogues against aromatase<br>(CYP19) and the EC <sub>50</sub> values of lead compounds to ERs (ER- $\alpha$ and ER- $\beta$ ) ..... | 97  |
| Table 13. Summary of structure-activity relationships .....  | 104 |

## LIST OF FIGURES

|  |    |
|--|----|
| Figure 1. Chemical structures of tamoxifen and its representative metabolites .....  | 18 |
| Figure 2. Chemical structures of <i>E</i> - and <i>Z</i> - norendoxifen .....  | 54 |
| Figure 3. Representative chromatograms of extracted samples.....   | 56 |
| Figure 4. Plasma concentrations of hydroxynorendoxifen in 10 breast cancer patients plasma samples .....   | 63 |
| Figure 5. Inhibition of cytochrome P450 enzymes by norendoxifen ( <i>E,Z</i> ).....  | 66 |
| Figure 6. Dixon plots of inhibition of CYP1A2, CYP2A6, CYP3A4, CYP3A5 and CYP2C19 by norendoxifen ( <i>E,Z</i> ) .....                                   | 67 |
| Figure 7. Inhibition of cytochrome P450 enzymes by hydroxynorendoxifen ( <i>E,Z</i> ) .....  | 69 |
| Figure 8. Dixon plots of inhibition of aromatase (CYP19), CYP1A2, CYP2A6, CYP3A4, CYP3A5, CYP2D6 and CYP2C19 by hydroxynorendoxifen ( <i>E,Z</i> ) ..... | 70 |
| Figure 9. Inhibition of aromatase (CYP19), CYP1A2, CYP3A4, CYP3A5 and CYP2C19 by <i>E</i> - and <i>Z</i> -norendoxifen. ....                             | 73 |
| Figure 10. Possible metabolic pathway involved in the production of hydroxynorendoxifen from tamoxifen.....  | 80 |
| Figure 11. Inhibition of cellular aromatase activity in MCF-7 cells by norendoxifen ( <i>E,Z</i> ) and hydroxynorendoxifen ( <i>E,Z</i> ).....           | 85 |
| Figure 12. Activity of norendoxifen and hydroxynorendoxifen to antagonize progesterone receptor (PGR) mRNA expression in MCF-7 cells.....                | 87 |

|  |     |
|--|-----|
| Figure 13. Anti-proliferative effects of norendoxifen ( <i>E,Z</i> ) and<br>hydroxynorendoxifen ( <i>E,Z</i> ) on human breast cancer cells..... | 88  |
| Figure 14. Chemical structures of norendoxifen analogues.....  | 95  |
| Figure 15. Activity of lead norendoxifen analogues to antagonize<br>progesterone receptor (PGR) mRNA expression in MCF-7 cells.....              | 101 |
| Figure 16. Summary of structure-activity relationships .....   | 103 |

## LIST OF ABBREVIATIONS

|          |   |
|----------|---|
| 3,4-DHT  | 3,4-dihydroxy-tamoxifen                             |
| 3-HT     | 3-hydroxy-tamoxifen                                 |
| 4'-HT    | 4'-hydroxy-tamoxifen                                |
| 4-HT     | 4-hydroxy-tamoxifen                                 |
| ADC      | antibody-drug conjugate                             |
| AI       | aromatase inhibitors                                |
| ATAC     | anastrozole, tamoxifen alone or in combination      |
| AUC      | area under curve                                    |
| BCIS     | breast carcinoma in situ                            |
| BCL2     | B-cell CLL/lymphoma                                 |
| BFC      | 7-benzyloxy-4-trifluoromethylcoumarin               |
| BIG 1-98 | Breast International Group 1-98                     |
| cAMP     | cyclic adenosine monophosphate                      |
| CARM1    | coactivator-associated arginine methyltransferase 1 |
| CCND1    | c-Myc and cyclin D1                                 |
| cDNA     | complementary deoxyribonucleic acid                 |
| CE       | collision energy                                    |

|       |                                   |
|-------|-----------------------------------|
| CEC   | 3-cyano-7-ethoxycoumarin          |
| CEP   | cell entrance potential           |
| CHC   | 3-cyano-7-hydroxycoumarin         |
| CV    | coefficient of variation          |
| CXP   | cell exit potential               |
| CYP   | cytochrome P450                   |
| CYP19 | aromatase                         |
| DBD   | DNA-binding domain                |
| DCIS  | ductal carcinoma in situ          |
| DDI   | drug-drug interaction             |
| DFS   | disease-free survival             |
| DM1   | mertansine                        |
| DP    | declustering potential            |
| DPH   | diphenylhydantoin                 |
| E1    | estrone                           |
| E2    | 17 $\beta$ -estradiol (estradiol) |
| E3    | estriol                           |
| EGFR  | epidermal growth factor receptor  |

|                  |   |
|------------------|---|
| EP               | entrance potential  |
| ER               | estrogen receptor   |
| EREs             | estrogen-responsive elements                                    |
| FBS              | fetal bovine serum  |
| FDA              | Food and Drug Administration                                    |
| HBD              | hormone-binding domain  |
| HC               | 7-hydroxycoumarin   |
| HER2             | human epidermal growth factor receptor 2                        |
| HFC              | 7-hydroxy-4-trifluoromethylcoumarin                             |
| HPLC-MS/MS       | high performance liquid chromatography-tandem mass spectrometry |
| HR               | hormone receptor  |
| IC <sub>50</sub> | half maximal inhibitory concentration                           |
| IDC              | invasive ductal carcinomas                                      |
| IES 1-98         | Intergroup Exemestane Study 1-98                                |
| IG <sub>50</sub> | half maximal cellular growth inhibitory concentration           |
| IHC              | immunohistochemical   |
| ILC              | invasive lobular carcinoma                                      |



|                  |                                     |
|------------------|-------------------------------------|
| IS               | internal standard                   |
| LCIS             | lobular carcinoma in situ           |
| LDL              | low-density lipoprotein             |
| LOB              | limit of blank                      |
| LOD              | limit of detection                  |
| LOQ              | limit of quantification             |
| MAPK             | mitogen-activated protein kinase    |
| MEM              | minimum essential medium            |
| MFC              | 7-methoxy-4-trifluoromethylcoumarin |
| MRI              | magnetic resonance imaging          |
| MRM              | MRM, multiple reaction monitoring;  |
| mTOR             | mammalian target of rapamycin       |
| <i>N,N</i> -DDMT | <i>N</i> -didesmethyl-tamoxifen     |
| <i>N</i> -DMT    | <i>N</i> -desmethyl-tamoxifen       |
| NOS              | not otherwise specified             |
| PCR              | polymerase chain reaction           |
| PD               | pharmacodynamics                    |
| PK               | pharmacokinetics                    |

|                  |  |
|------------------|--|
| PR               | progesterone receptor                            |
| PRMT1            | protein arginine <i>N</i> -methyltransferase 1   |
| RHA              | RNA helicase A                                   |
| RNA              | ribonucleic acid                                 |
| RT               | retention time; MW, molecular weight             |
| SD               | standard deviation                               |
| SERM             | selective estrogen receptor modulators           |
| SRC              | steroid receptor coactivator                     |
| SSRI             | selective serotonin reuptake inhibitor           |
| SW1/SNF          | SWItch/Sucrose NonFermentable                    |
| SWOG             | Southwest Oncology Group                         |
| T-DM1            | trastuzumab emtansine                            |
| TFF1/pS2         | trefoil factor 1/presenelin-2                    |
| TNBC             | triple-negative breast cancer                    |
| $\alpha$ -HN-DMT | $\alpha$ -hydroxy- <i>N</i> -desmethyl-tamoxifen |
| $\alpha$ -HT     | $\alpha$ -hydroxy-tamoxifen                      |

## **CHAPTER 1: INTRODUCTION**

### **1.1 Breast cancer and its classification**

Breast cancer is the most prevalent cancer diagnosed in women and the second most common cancer worldwide after lung cancer. Breast cancer remains a major threat to the lives of women, even though the mortality caused by breast cancer has declined in the past two decades.

Breast cancer is a heterogeneous disease characterized by its variable morphological features, different molecular subtypes and diverse clinical outcomes. According to different scientific criteria and/or clinical practices, three classification schemes of breast cancer were established: 1) immunohistochemical (IHC) classification based on biomarkers; 2) molecular classification based on gene expression profiling; 3) histopathological classification based on morphological features<sup>1</sup>.

The first classification scheme, IHC classification, was established based on well-defined biomarkers, which played important roles for the development and invasion of breast cancer. Using this classification scheme, breast cancers are grouped into subtypes based on the hormone receptors (HRs) status and the expression status of human epidermal growth factor receptor 2 (HER2). Breast cancers that contain either estrogen receptors (ERs) or progesterone receptors (PRs) are HR-positive, which depends on estrogen signaling or progesterone signaling or both. Otherwise, they are HR-negative breast cancer. Breast cancers that overexpress HER2 or have extra copies of the HER2 gene are called HER2-

positive. Otherwise, they are HER2-negative breast cancer. Breast cancers that don't have HRs or overexpress HER2 are classified as triple-negative.

Currently, IHC classification along with clinical pathologic variables, such as nodal involvement, tumor size, histologic type, tumor grade, and surgical margins, are commonly used to select treatment and to predict disease prognosis<sup>2</sup>. Therefore, IHC classification has been widely applied by diagnostic laboratories to classify breast cancer types and to help choose the optimal treatment approach<sup>3</sup>.

The second classification scheme, molecular classification, was established based on gene expression profiling using hierarchical clustering of groups of genes based on the similarity of gene expression patterns. Using this classification scheme, breast cancers are classified in five subtypes: luminal A, luminal B, HER2-positive, basal-like and claudin-low.

Although this classification scheme of breast cancers has not been implemented clinically for patient management, it links the molecular biology of breast cancers and breast cancer cells' behavior in the five subtypes. In this context, molecular classification has prognostic value and may be predictive of response to chemotherapy.

The third classification scheme, histopathological classification, was established based on the morphological features of the tumors. Using this classification scheme, breast cancers can be divided into in situ carcinoma and invasive (infiltrating) carcinoma. Breast carcinoma in situ (BCIS) is further sub-classified as either lobular carcinoma in situ (LCIS) or ductal carcinoma in situ

(DCIS). LCIS is considerably less common than its DCIS counterpart and encompasses a heterogeneous group of tumors. DCIS has traditionally been further subclassified based on the architectural features of the tumor which has given rise to 5 subtypes: Comedo, Cribiform, Micropapillary, Papillary and Solid<sup>4</sup>.

A major drawback of this classification scheme is that about 70%-80% of the all breast cancers will belong to either one of the two major categories, namely invasive ductal carcinomas, not otherwise specified (IDCs NOS), or invasive lobular carcinoma (ILC). Hence, this classification scheme has minimal prognostic and predictive implications and its clinical utilization is quite modest<sup>5</sup>.

## **1.2 Breast cancer management and treatment**

Currently, clinical breast examination, mammography and ultrasound are the standards for the diagnosis of breast cancer. While, multidisciplinary management including clinical, radiological, pathological and core biopsy has been applied for the diagnosis of breast cancer<sup>6</sup>. Magnetic resonance imaging (MRI) has been shown to be useful in many cases to determine malignancy extent<sup>6</sup>.

Usually, wide local excision breast conserving surgery is preferred. Mastectomy is mainly recommended when wide local excision breast conserving surgery is not suitable due to tumor size or multifocal disease, or for the patients requesting mastectomy. All the patients who received breast conserving surgery are also treated with breast radiotherapy. Chemotherapy or endocrine therapy

may be given before breast conserving surgery to reduce tumor size and facilitate breast conservation.

After breast surgery, an adjuvant therapy plan is commonly decided by the pathology report including histological grade, HRs expression (ERs and PRs) status and HER2 expression status. Endocrine therapy for the treatment of ER-positive breast cancer can be divided into three classes: selective estrogen receptor modulators (SERMs), selective estrogen receptor down-regulators (SERDs) and aromatase inhibitors (AIs). Both pre-menopausal and post-menopausal women can be treated with SERMs for the ER-positive breast cancer. The US Food and Drug Administration (FDA) has approved three SERMs (tamoxifen, raloxifene and toremifene) for the prevention or treatment of ER-positive breast cancer, among them, tamoxifen has become the most widely used SERM. Only one SERD (fulvestrant) was approved by US FDA for the treatment of ER-positive metastatic breast cancer in post-menopausal women. Currently, the US FDA approved aromatase inhibitors (AIs), including letrozole, anastrozole and exemestene, are superior to tamoxifen for the treatment of ER-positive breast cancer in post-menopausal women.

Besides the available endocrine therapy, for patients with HER2-positive breast cancer, HER2-targeted medications, including trastuzumab, pertuzumab, lapatinib and trastuzumab emtansine (T-DM1), have been shown to have survival benefits, even though these drugs have different mechanisms. Among them, trastuzumab and pertuzumab are monoclonal antibodies that target the HER2 extracellular domain and block its activation. Lapatinib is a small-molecule kinase

inhibitor targeting HER2 and epidermal growth factor receptor (EGFR). T-DM1 is an antibody-drug conjugate (ADC), in which the monoclonal antibody trastuzumab is linked to a small-molecule microtubule inhibitor mertansine (DM1). Since patients with triple-negative breast cancer (TNBC) lack expression of HRs and do not exhibit amplification of HER2, they will not benefit from SERMs, AIs or HER2-targeted drugs. Until now, there is no approved targeted therapy for the treatment of TNBC. Chemotherapy, combined with surgery and radiation therapy, is the only treatment option for TNBC.

### **1.3 Estrogen receptor-positive cancer and estrogen signaling**

More than two thirds of all diagnosed breast cancers are estrogen receptor (ER)-positive and are dependent on estrogen signaling. ER has two isoforms, ER- $\alpha$  and ER- $\beta$ , belonging to a superfamily of nuclear hormone receptors that function as transcriptional factors when bound by ligands. Estrogenic actions in both normal breast cells and breast cancer cells are mediated primarily by ER- $\alpha$ . Since ER- $\alpha$  is involved in the pathogenesis of breast cancer, it has been considered as a target for the treatment of breast cancer for a long time<sup>7</sup>.

ER- $\alpha$  is encoded by a gene localized on chromosome 6q24-27, and ER- $\beta$  is encoded by a gene located on chromosome 14q21-22. ER- $\alpha$  has 595 amino acids, while ER- $\beta$  has 530 amino acids. Both ER- $\alpha$  and ER- $\beta$  have similar structures and their functional domains can be divided into five regions: A/B, C, D, E and F domains. Among these five domains, C domain is a DNA-binding

domain (DBD) for DNA recognition and binding, whereas E domain is a hormone-binding domain (HBD) or ligand-binding domain for ligand binding. The DBDs of ER- $\alpha$  and ER- $\beta$  are highly conserved with a 95% homology, while, their HBDs are less conserved with only 53% homology. In addition, there are two distinct transactivation domains located in A/B and E regions, termed activation factor-1 (AF-1) and activation factor-2 (AF-2), respectively. Transcriptional activation mediated by ERs is stimulated through AF-1 and AF-2 (hormone-dependent), among them, AF-1 is hormone-independent, while AF-2 is hormone-dependent<sup>8</sup>.

Estrogens play important roles in sex determination, fertility, pregnancy, immune response, bone formation and cardiovascular system. Estrogens share a common four-ring chemical structure with cholesterol, because they are derivatives of cholesterol, which can be converted into progesterones, then into androgens and finally into estrogens by a series of enzymatic reactions<sup>9</sup>. There are three primary types of estrogens: estrone (E1), estradiol (17 $\beta$ -estradiol, E2) and estriol (E3). These estrogens are named based on how many hydroxyl groups they have. E2 has the highest binding affinity for ERs, followed by E1 and then E3<sup>10</sup>. E2 is converted from testosterone and it is the major estrogen in pre-menopausal women. E1 is converted from androstenedione and it is the major estrogen in post-menopausal women. During pregnancy, E3 is converted from 16- $\alpha$ -hydroxyandrostenedione and it is the major estrogen in pregnant women.

ER- $\alpha$  is usually in its inactive state by forming a complex with proteins including heat shock protein 70, heat shock protein 90, cyclophilin 40, FKBP51



and FKBP52. Estrogens diffuse through cellular plasma and then nuclear members and finally bind to ER- $\alpha$  through its HBD<sup>11</sup>. This binding leads to the dissociation of inactive complex and then activates the conformational changes for dimerization. Binding of estrogens to ER- $\alpha$  can trigger recruitment of various cellular factors in a complex that alters chromatin structure and facilitates binding of RNA polymerase II transcriptional activation<sup>11</sup>. Therefore, estrogen-ER complex functions as a transcriptional activator, which is able to induce promoter gene expression.

The dimeric ER- $\alpha$ s bind to DNA with high affinity through their DBD at specific sites called estrogen-responsive elements (EREs) in the promoter region of estrogen-responsive genes. Then, coactivators like members of the p160 steroid receptor coactivator (SRC) family (SRC-1, Tif-2/GRIP1 and SRC-3/AIB1) can interact with ER- $\alpha$  via common  $\alpha$ -helical peptide sequences. The  $\alpha$ -helical peptide sequences are hydrophobic and amphipathic motif LXXLL (L = leucine and X = any amino acid). The SRC family interact with the histone acetyltransferases, cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB)-binding protein (CBP), and p300, which acetylate, and with coactivator-associated arginine methyltransferase 1 (CARM1) and protein arginine *N*-methyltransferase 1 (PRMT1), which methylate histones within the nucleosomes<sup>11</sup>. An RNA helicase A (RHA), and an ATP-dependent chromatin remodeling complex, SWItch/Sucrose NonFermentable (SW1/SNF), are recruited. The resultant coactivator complex modifies the nucleosomes and alters the surrounding chromatin to allow access to the activating transcription factor

proteins, TATA-binding protein, and RNA polymerase II machinery, and transcription ensues<sup>12</sup>.

Well-known estrogen-responsive genes include progesterone receptor (PR), trefoil factor 1/presenelin-2 (TFF1/pS2), B-cell CLL/lymphoma (BCL2), cathepsin D, cyclin D1, c-Myc and cyclin D1 (CCND1) genes.

#### **1.4 Selective estrogen receptor modulators (SERMs)**

Selective estrogen receptor modulators (SERMs) are a diverse group of compounds that function as agonists or antagonists for ERs with a target tissue-specific profile. SERMs are used for various indications, including treatment of breast cancer, osteoporosis, and menopausal symptoms. When SERMs act as agonists, their mechanisms of action include binding to ERs, then triggering the dimerization of ERs, binding to DNA and finally activating transcription after recruiting a series of coactivators. When SERMs act as antagonists, their mechanisms of action include binding to ERs and favoring a corepressor binding conformation instead of coactivator binding, thus blocking the activation of estrogen-responsive genes<sup>13</sup>.

The tissue-specific profile of SERMs may be caused by several of factors:

- 1) SERMs have different binding affinities for ER subtypes;
- 2) ER subtypes are differently expressed in target tissues;
- 3) co-factors are differently expressed in target tissues;
- 4) the binding of SERMs may induce different ERs conformational changes that further influence the dimerization and binding to co-factors.

Currently, US FDA approved SERMs can be divided into two categories based on their chemical structures: 1) triphenylethylene derivatives (tamoxifen, toremifene, ospemifene and clomiphene); 2) benzothiophene derivatives (raloxifene and bazedoxifene).

#### **1.4.1 Triphenylethylene derivatives**

Tamoxifen (developed by AstraZeneca) is a first generation SERM developed in 1970s and was approved by US FDA in 1977. It is the first-line endocrine therapy as adjuvant therapy for both early and metastatic (advanced) ER-positive breast cancer, and it has also been approved by US FDA for the prevention of breast cancer development in high-risk women. Tamoxifen acts as an ER antagonist in breast cancer cells, while, tamoxifen displays ER agonistic effects in uterus, bone, and vascular venous system. The different actions of tamoxifen in various tissues may be caused by the various expressions of the co-factor proteins. For example, SRC1, a co-factor promoted by tamoxifen, is highly expressed in uterine cells. However, it is lowly expressed in breast cancer cells. Treatment of tamoxifen increase the risks of several side effects including endometrial cancer, hot flashes, thromboembolic events, ocular toxicity, stroke and pulmonary embolism<sup>14</sup>. The occurrence of these side effects can be attributed to the partial estrogen agonistic effects of tamoxifen, for example, the increased risks of endometrial cancer and thromboembolic events in the uterus and vascular venous system. Several studies have shown that the use of

tamoxifen is associated with increased level of systemic estrogen, however, the mechanism is still unknown<sup>15,16</sup>.

Toremifene (developed by GTx Inc.) is a tamoxifen analogue by the presence of a chlorine atom at the 4 position, and its preclinical and clinical activities are very similar to those of tamoxifen. It has been approved by US FDA as a treatment of advanced (metastatic) ER-positive breast cancer in post-menopausal women. Because the similarities between toremifene and tamoxifen, the functional mechanisms and safety profiles of toremifene is similar to tamoxifen<sup>17</sup>.

Ospemifene (developed by Shionogi Inc.) is a tamoxifen analogue with a chlorine atom at the 4 position but without the 2-(dime-thylamino) ethoxy region. It has been approved by US FDA in 2013 for the treatment of dyspareunia (usually moderate to severe). Dyspareunia is associated with vulvar and vaginal atrophy because of menopause. Similar to tamoxifen and toremifene, ospemifene has anti-estrogenic effect in breast tissue, estrogenic effect in bone and partial ER agonist effects in uterine and vaginal tissues. Ospemifene is the only SERM with nearly full estrogen agonistic effect on the vaginal epithelium, and it has relatively weaker estrogen agonistic effects on endometrium. This unique feature of ospemifene makes it the only SERMs for the treatment of dyspareunia. The treatment of ospemifene is associated with side effects including hot flashes, vaginal discharge, muscle spasms<sup>18</sup>.

Clomiphene (developed by Sanofi-aventis U.S. LLC, now Sanofi) has a different indication from the other SERMs. It has a similar chemical structure to

tamoxifen and was approved by US FDA in 1967 for the treatment of ovulatory dysfunction. Clomiphene remains a first-line drug for the treatment of anovulatory infertility<sup>19</sup>.

#### **1.4.2 Benzothiophene derivatives**

Raloxifene (developed by Eli Lilly & Co) is a second generation SERM with a polyhydroxy phenol benzothiophene that has different tissue-specific effects compared to tamoxifen. It has been approved by US FDA for the treatment and prevention of post-menopausal women's osteoporosis and for the reduction in risk of invasive breast cancer in post-menopausal women with osteoporosis<sup>20</sup>.

Bazedoxifene (developed by Wyeth Pharmaceuticals, Inc., a wholly owned subsidiary of Pfizer, Inc.) is a third generation SERM with a core binding domain consisting of a 2-phenyl-3-methyl indole and a hexamethylenediamine ring at the side chain terminus. It has been approved for the treatment of vasomotor symptoms due to menopause and for the prevention of post-menopausal women's osteoporosis<sup>21</sup>.

#### **1.5 Selective estrogen receptor down-regulators (SERDs)**

Selective estrogen receptor down-regulators (SERDs) are distinguishable from SERMs pharmacologically. SERDs function as pure ER antagonists, which can lead to impaired dimerization, increased ER turnover and disrupted nuclear localization<sup>22</sup>.

### **1.5.1 Fulvestrant**

Fulvestrant (developed by AstraZeneca) is the only SERD approved by US FDA, and its indication is for the treatment of ER-positive metastatic breast cancer in post-menopausal women. Binding of fulvestrant to ERs will lead to ER degradation and loss of the ER protein. Both *in vitro* and *in vivo* studies have shown that after binding of fulvestrant, the ER turnover is increased and nuclear localization is disrupted with a reduced number of detectable ER molecules<sup>23</sup>.

## **1.6 Aromatase and aromatase inhibitors**

### **1.6.1 Aromatase**

Aromatase (CYP19) is encoded by CYP19A1 gene residing on chromosome 15q21 in humans and it is the sole member. Aromatase is the only enzyme responsible for the production of E2 from testosterone, E1 from androstenedione and E3 from 16- $\alpha$ -hydroxyandrostenedione by demethylation and subsequent aromatization. Specifically, aromatase catalyzes the conversion of C19-androgens to C18-estrogens through a 3-step reaction that sequentially generates 19-hydroxy and 19-aldehyde intermediates before aromatase<sup>24</sup>.

Aromatase belongs to the cytochrome P450 (CYP) superfamily, which plays dominant roles in drug metabolism. This superfamily includes various CYP isoforms, such as CYP1A2, CYP2A6, CYP3A4, CYP3A5, CYP2C19 and CYP2D6. In human liver, CYP1A2 accounts for 13% of the CYP content and catalyzes the primary metabolic route for a number of important drugs, including caffeine, clozapine, flutamide, lidocaine, olanzapine and zolmitriptan<sup>25</sup>. CYP2A6

is a major CYP in human liver, specifically involved in the oxidative metabolism of nicotine. It is also involved in the metabolism of pharmaceutical agents such as methoxyflurane, halothane, losigamone, letrozole, valproic acid, disulfiram and fadrozole<sup>26,27</sup>. CYP3A4 and CYP3A5 account for about 50% of the CYP content and are the predominant CYP contributors to metabolism in human liver, accounting for 40-60% of the oxidative metabolism of marketed drugs<sup>28</sup>. CYP2C19 can metabolize 10-15% of drugs on the market, including omeprazole, mephenytoin, proguanil and diazepam<sup>29</sup>. CYP2D6 can metabolize about 25% of drugs in current clinical use, including tamoxifen<sup>30</sup>. Any proposed new drug that undergoes significant metabolism by CYP superfamily should be evaluated for CYPs inhibition and further for drug-drug interactions (DDIs).

However, unlike most of the CYPs, aromatase is not highly expressed in healthy human livers and hepatic aromatase activity is minimal. In humans, aromatase is expressed in ovaries as well as numerous extragonadal tissues including testes, placenta, mesenchymal cells of adipose tissue (but not the lipid-filled mature adipocytes), osteoblasts, chondrocytes of bone, vascular smooth muscle, endothelium and brain<sup>31</sup>. In pre-menopausal women, aromatase is mainly present in ovaries, while, in the post-menopausal women, adipose is the largest tissue containing aromatase. Therefore, aromatase activity is high in the ovaries of pre-menopausal women, while, aromatase activity is high in the adipose tissue of post-menopausal women. In addition, aromatase expression and thus aromatase activity have been shown to be greatly increased in malignant but not normal breast tissue.

The crystal structure of human aromatase has demonstrated that it is a heterodimer made up of a CYP aromatase and a ubiquitous NADPH CYP reductase. The catalytic domain of aromatase contains a heme group in a steroid binding site<sup>32</sup>.

### **1.6.2 Aromatase inhibitors**

The conversion from peripheral androgens to estrogens by aromatase is the primary source of estrogen production in post-menopausal women and in women, who have reduced or eliminated ovarian function after a pathological change or medical intervention. Therefore, aromatase has become an important target for the treatment of ER-positive breast cancer in post-menopausal women. Aromatase inhibitors (AIs) were developed to reduce the peripheral estrogen production in post-menopausal women by blocking aromatase activity and in turn inhibit the tumor growth.

Currently, the US FDA has approved three third generation AIs: letrozole, anastrozole and exemestane. These three AIs can be classified as steroidal (exemestane) and non-steroidal (letrozole and anastrozole) compounds. Steroidal AIs are analogues of natural aromatase substrates, testosterone and androstenedione. They can bind competitively to the binding site of aromatase, forming tight, irreversible covalent bonds that result in permanent enzyme inactivation. Reactivation of aromatase activity is dependent on further synthesis of aromatase. Non-steroidal AIs interact with the heme moiety inside the binding site of aromatase and in turn inhibit steroidal aromatization. Thus, sustained



aromatase inhibition depends on the continuous presence of drug. In vivo tracer studies indicate that third generation AIs may inhibit total systemic estrogen level by 98%, there are several studies indicating systemic estrogen level to be sustained at 20-40% of pre-treatment levels on therapy<sup>33</sup>. In post-menopausal women with advanced disease, both steroidal and non-steroidal AIs have shown good clinical efficacy without cross-resistance between the two groups, and similarly acceptable short-term toxicity profiles<sup>34</sup>.

Letrozole (developed by Novartis Pharmaceuticals Corp.) is a non-steroidal AI approved by US FDA for the adjuvant treatment of early ER-positive breast cancer in post-menopausal women or extended adjuvant treatment of early breast cancer in post-menopausal women after five years of adjuvant tamoxifen treatment. Letrozole has also been approved for the adjuvant treatment of metastatic breast cancer in post-menopausal women<sup>35</sup>.

Anastrozole (developed by AstraZeneca) is also a non-steroidal AI approved by the US FDA for the adjuvant treatment of early ER-positive breast cancer in post-menopausal women. It has also been approved as first-line treatment of ER-positive or unknown locally advanced (metastatic) breast cancer and second-line adjuvant treatment of metastatic breast cancer with disease progression after tamoxifen therapy<sup>36</sup>.

Exemestane (developed by Pfizer Inc.) is a steroidal AI approved by the US FDA for the adjuvant treatment of early ER-positive breast cancer in post-menopausal women who have received two to three years of tamoxifen. It has also been approved for the treatment of advanced breast cancer in post-

menopausal women whose disease has progressed following tamoxifen therapy<sup>37</sup>.

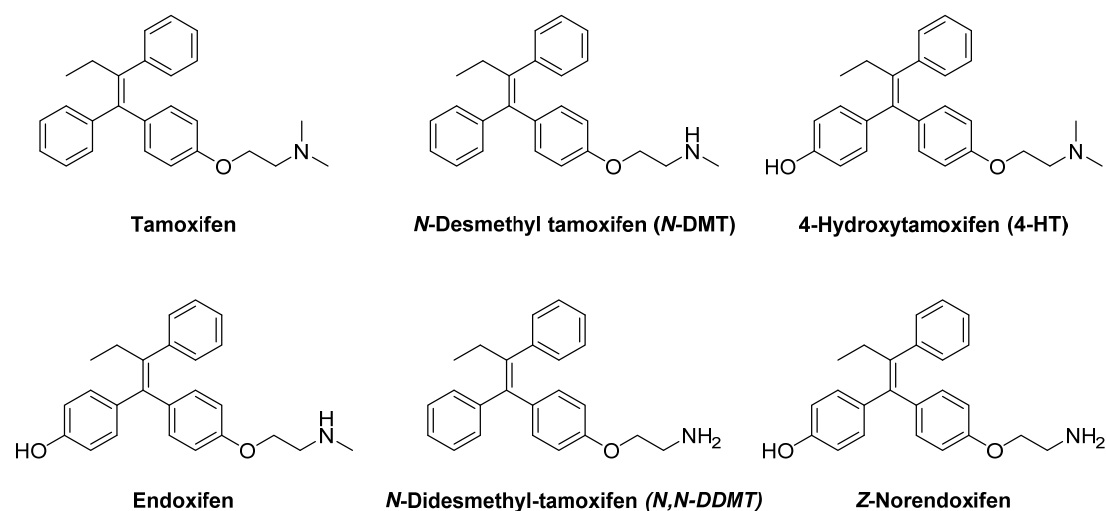
### **1.7 Tamoxifen and its metabolites**

Over the past 30 years, tamoxifen, a SERM, has been the most widely used drug for the adjuvant treatment and prevention of both early and advanced (metastatic) ER-positive breast cancer. Tamoxifen itself has low binding affinity to ER and thus is considered to be a pro-drug functioning through its active metabolites. The metabolism of tamoxifen has a complex profile involving both active and inactive metabolites by both phase I and phase II liver enzymes. The metabolites of tamoxifen can be classified as primary and secondary metabolites based on their sequence and amount. According to an *in vitro* extensive study by Dr. Desta, the primary metabolites of tamoxifen include *N*-desmethyl-tamoxifen (*N*-DMT), 4-hydroxy-tamoxifen (4-HT),  $\alpha$ -hydroxy-tamoxifen ( $\alpha$ -HT), 3-hydroxy-tamoxifen (3-HT) and 4'-hydroxy-tamoxifen (4'-HT). The secondary metabolites of tamoxifen include 4-hydroxy-*N*-desmethyl-tamoxifen (endoxifen), *N*-didesmethyl-tamoxifen (*N,N*-DDMT), 3,4-dihydroxy-tamoxifen (3,4-DHT) and  $\alpha$ -hydroxy-*N*-desmethyl-tamoxifen ( $\alpha$ -HN-DMT)<sup>38</sup>. The primary major metabolite of tamoxifen, *N*-DMT, is an inactive compound with a weak anti-estrogen effect and is mainly formed via CYP3A4/5 (minor formation via CYP2C9, CYP2C19 and CYP2B6). The minor primary metabolite of tamoxifen, 4-HT, is the first characterized active metabolite possessing high binding affinity for ERs and potent activity in suppressing estrogen-responsive cell growth and estrogen-

responsive genes<sup>39</sup>. The formation of 4-HT is mainly via CYP2D6 and other CYPs including CYP2C9, CYP2C19 and CYP3A play less important roles. Other minor primary metabolites of tamoxifen, including  $\alpha$ -HT, 3-HT and 4'-HT, are mainly formed by CYP3A4/5, CYP3A4/5 and CYP2B6.

Besides these primary metabolites, a secondary tamoxifen metabolite, endoxifen, has been shown to have potent activity similar to 4-HT with respect to ERs binding affinity, inhibition of estrogen-responsive cell growth and estrogen-responsive gene expression<sup>40</sup>. Since clinical data has shown that the plasma concentration of endoxifen is 5- to 7-fold higher than that of 4-HT in patients, endoxifen is considered the most important active metabolite of tamoxifen<sup>41</sup>. Endoxifen is formed from *N*-DMT and 4-HT mainly via CYP2D6 and CYP3A4/5, respectively.

Figure 1 shows the chemical structures of tamoxifen and its important metabolites, including tamoxifen, *N*-DMT, 4-HT, endoxifen, *N,N*-DDMT and *Z*-norendoxifen. Table 1 shows the plasma concentrations of tamoxifen, *N*-DMT, 4-HT and norendoxifen reported by Dr. Flockhart's and Dr. Schwab's groups<sup>42-44</sup>.



**Figure 1. Chemical structures of tamoxifen and its representative metabolites**

**Table 1. Plasma concentrations of tamoxifen, *N*-DMT, 4-HT and norendoxifen**

|                     | Average plasma concentration (nM) |
|---------------------|-----------------------------------|
| <b>Tamoxifen</b>    | <b>372.5</b>                      |
| <b><i>N</i>-DMT</b> | <b>653.4</b>                      |
| <b>4-HT</b>         | <b>9.5</b>                        |
| <b>Endoxifen</b>    | <b>78.0</b>                       |
| <b>Norendoxifen</b> | <b>3</b>                          |

In 20014, Dr. Lien's group reported a study for target tissue concentration of tamoxifen and its metabolites. This study includes 108 serum samples, 62 breast cancer tissue sample and 52 normal breast tissue samples for analysis of tamoxifen and its metabolite concentrations. The median (mean) concentration of tamoxifen in subjects taking 20 mg/day dosage is 83.6 (77.1) ng/ml. The median concentrations of tamoxifen and *N*-DMT in the normal breast tissues and breast cancer tissues were 5-11 times higher than those observed in serum. Unlike 4-HT, tamoxifen, *N*-DMT and *N,N*-DDMT in serum were related to their levels in normal breast tissue and breast cancer tissues. For 4-HT, in normal breast tissue there were lower correlations between 4HT and tamoxifen, *N*-DMT, and *N,N*-DDMT. While in tumor tissue there were high correlations<sup>45</sup>.

Other enzymatic conversions from primary metabolites to secondary metabolites include: *N*-DMT can be metabolized to *N,N*-DDMT and  $\alpha$ -NH-desmethyl-tamoxifen predominantly via CYP3A4/5; 4-HT, which can be metabolized to 3,4-DHT via CYP3A4/5<sup>38</sup>.

Besides the ER antagonistic activity of endoxifen, it has also been shown to inhibit recombinant human aromatase with an IC<sub>50</sub> value of 4000 nM via a non-competitive mechanism *in vitro*. Previous studies by our group identified another metabolite of tamoxifen, *N,N*-didesmethyl-4-hydroxy-tamoxifen, which was the demethylated product of endoxifen and thus was named norendoxifen (Nor)<sup>46</sup>. Norendoxifen has been shown to inhibit recombinant human aromatase with a *Ki* value of 70 ± 9 nM via a competitive mechanism<sup>47</sup>. Further studies have shown

that norendoxifen can bind to ER- $\alpha$  and - $\beta$  with EC<sub>50</sub> values of  $27 \pm 5$  nM and  $35 \pm 17$  nM, respectively<sup>48</sup>.

As you may see, the primary metabolites of tamoxifen are likely to be further metabolized via demethylation or hydroxylation. Secondary metabolites are less likely to be further metabolized.

## **1.8 Comparison of SERMs, SERDs and AIs for the treatment of ER-positive breast cancer**

Since Tamoxifen is the most widely used SERM for the treatment of ER-positive breast cancer, we will use tamoxifen as a representative of SERMs.

### **1.8.1 Tamoxifen versus AIs**

Many studies have shown that the use of an AI as primary adjuvant therapy is superior to tamoxifen for the treatment of ER-positive breast cancer in post-menopausal women. Compared to tamoxifen, AIs are also associated with fewer serious side effects<sup>49</sup>.

A randomized clinical trial comparing tamoxifen with letrozole is the “Breast International Group (BIG) 1-98” trial. BIG 1-98 is a double-blind clinical trial involving 8010 patients randomly assigned to four treatment arms: 1) 5 years of tamoxifen; 2) 5 years of letrozole; 3) 2 years of tamoxifen + 3 years of letrozole; 4) 2 years of letrozole + 3 years of tamoxifen<sup>50,51</sup>. The results have shown that 5 years of letrozole significantly prolonged disease-free survival

(DFS), overall survival (OS) and time to distant recurrence (TDR) in comparison with 5 years of tamoxifen<sup>52</sup>.

Randomized clinical trials comparing tamoxifen with anastrozole include the “Anastrozole, Tamoxifen Alone or in Combination (ATAC)” trial, “ARNO 95” trial and “ITA” trial. The ATAC trial is a double-blind clinical trial involving 9366 subjects randomly assigned to three treatment arms: 1) 5 years of tamoxifen (reference arm); 2) 5 years of anastrozole (first experimental arm); 3) 5 years of combined tamoxifen/anastrozole (second experimental arm). The results have shown that 5 years of anastrozole significantly improved DFS, improved time to recurrence and reduced distant metastases in comparison with 5 years of tamoxifen<sup>53</sup>. ITA is a randomized clinical trial involving 448 patients (received 2-3 years of adjuvant tamoxifen treatment) assigned to two treatment arms: 1) 2-3 years of continued tamoxifen (5 years of total duration) or 2) 2-3 years of anastrozole (5 years of total duration). The results have shown that switching to anastrozole significantly prolonged DFS and time to recurrence by months. Even though the OS in anastrozole arm was longer than that in continued tamoxifen arm, the difference was not statistically significant (p-value=0.1)<sup>54,55</sup>. ARNO 95 (very similar to ITA) is a randomized clinical trial involving 969 patients (received 2 years adjuvant tamoxifen treatment) assigned to 2 treatment arms: 1) 3 years of continued tamoxifen or 2) 3 years of anastrozole. The results have shown that switching to anastrozole significantly prolonged DFS, prolonged OS and reduced disease recurrence in comparison with continuing tamoxifen treatment<sup>56,57</sup>.

Randomized clinical trials comparing tamoxifen with exemestane include the “Intergroup Exemestane Study (IES)” trial, and “TEAM” trial. IES is a double-blind clinical trial involving 4742 patients (received 2-3 years adjuvant tamoxifen treatment) randomly assigned to two treatment arms: 1) 2-3 years of continued tamoxifen (5 years of total duration) or 2) 2-3 years of exemestane (5 years of total duration). The results have shown that switching to exemestane significantly prolonged DFS. There was no significant difference for OS between the two arms<sup>58-60</sup>. TEAM is a randomized clinical trial involving 9779 patients assigned to two arms: 1) 5 years of exemestane or 2) 5 years of tamoxifen followed by exemestane. There was no significant difference for DFS between the two arms<sup>61</sup>.

### **1.8.2 Tamoxifen versus fulvestrant**

The first randomized clinical trial comparing tamoxifen with fulvestrant was published in 2004<sup>62</sup>. This trial is a double-blind clinical trial involving 587 patients assigned to two treatment arms: 1) fulvestrant arm or 2) tamoxifen arm with a median follow-up of 14.5 months. There was no significant difference for time to progression (TTP) between the two arms<sup>62</sup>.

### **1.8.3 Fulvestrant versus AI**

To date, only one AI, exemestane, was compared with fulvestrant to assess treatment efficacy in the clinical trials. Randomized clinical trials comparing fulvestrant with exemestane include the “Evaluation of Faslodex



versus Exemestane Clinical Trial (EFFECT)” trial and “Study of Faslodex, Exemestane and Arimidex (SoFEA)” trial. EFFECT is a double-blind clinical trial involving 693 patients randomly assigned to two treatment arms: 1) fulvestrant arm or 2) exemestane arm with a median follow-up of 13 months. The results have shown that there was no significant difference for TTP or clinical benefit rate between the two arms<sup>63,64</sup>. SoFEA is a randomized clinical trial involving 723 patients assigned to three treatment arms: 1) fulvestrant + anastrozole; 2) fulvestrant + placebo; 3) exemestane alone. The results have shown that there was no significant difference for progression-free survival (PFS) between fulvestrant + placebo arm and exemestane alone arm<sup>65</sup>.

## **1.9 Side effects of SERMs, SERDs and AIs for the treatment of ER-positive breast cancer**

The treatment effects of ER-positive breast cancer by both SERMs and AIs have been limited by side effects, which reduce the quality of life and compromise compliance. Both SERMs and AIs are associated with a number of side effects. Here, tamoxifen will be used as a representative of SERMs again.

### **1.9.1 Side effects of tamoxifen**

Tamoxifen is associated with increased risks of menopausal symptoms (including hot flashes and atrophic vaginitis), endometrial cancer, thromboembolic events, gynecologic symptoms (including vaginal dryness,

vaginal discharge and ovarian cysts), irregular menses, ocular toxicity, thrombocytopenia, and leukopenia<sup>66</sup>.

Menopausal symptoms are the most common side effects associated with tamoxifen, and they are more common in pre-menopausal women. Of the menopausal symptoms, hot flashes are the most frequent side effect affecting at least 50% of women taking tamoxifen. Since tamoxifen has partial estrogenic activity in some tissues (such as endothelium), it can increase the risk of side effects including endometrial cancer, endometrial hyperplasia, endometrial thickness, and ovarian cysts. The most serious side effect of tamoxifen is its potential of increasing the risk of endometrial cancer. Thromboembolic events, including deep venous thrombosis and pulmonary embolism, are uncommon but serious side effects caused by tamoxifen with an estimated incidence of 1.7% to 8.4%<sup>67</sup>.

Even though the partial estrogenic effects of tamoxifen increase the risk of side effects mentioned previously, these partial estrogenic activities prevent estrogen depletion which reduces the risk of other side effects, including bone fracture and muscle pain<sup>68</sup>.

### **1.9.2 Side effects of fulvestrant**

Common side effects associated with fulvestrant include gastrointestinal disturbances, hot flashes, ischemic cardiovascular disorder, joint disorders, thromboembolic events, urinary tract infection, vaginitis and weight gain<sup>69</sup>.

Interestingly, the side effects induced by fulvestrant seem very similar to combined side effects induced by AIs and tamoxifen, respectively.

### **1.9.3 Side effects of AIs**

There do not appear to be any differences in safety profiles of the third generation AIs (letrozole, anastrozole and exemestane), even though they have different chemical structures and distinct modes of actions. Generally, compared to tamoxifen, AIs have been shown to have enhanced safety profiles with less increased rates of serious side effects. However, long-term adherence of AIs is compromised by their side effects. AIs are associated with increased risks of musculoskeletal symptoms (including bone loss, arthralgia and myalgia), menopausal symptoms (including vaginal dryness, urinary problems and sexual problems), hot flashes and acne <sup>70</sup>.

Musculoskeletal symptoms are the most undesirable side effects associated with AIs with an incidence rate of 36%. Among these symptoms, bone loss is a predictable side effect because of estrogen deprivation by AIs, while, the pathologies of arthralgia (joint pain) or myalgia (muscle pain) were still unknown, and they were thought to be due to estrogen deprivation <sup>71</sup>.

Adjuvant clinical trials of AIs suggested that AIs are associated with increased risks of menopausal symptoms (including vaginal dryness, urinary problems and sexual problems). The cause of sexual problems is thought to be multifactorial <sup>72</sup>.

The mechanism of hot flashes caused by AIs is still unknown, but hot flashes remain a significant adverse effect in all the adjuvant clinical trials of AIs, with about 37% incidence rate. Even though hot flashes are not considered a severe side effect, long-term patient compliance may be decreased by increased intensity<sup>72</sup>.

The estrogen depriving effects of AIs may disturb lipid metabolism, have an adverse effect on blood lipids and in turn increase the risk of cardiovascular diseases. Several adjuvant clinical trials of AIs have shown an increased level of total serum cholesterol, low-density lipoprotein (LDL) cholesterol, apolipoprotein B, and serum-lipid risk ratios for cardiovascular disease<sup>73</sup>.

### **1.10 Tumor resistance to SERMs, SERDs and AIs for the treatment of ER-positive breast cancer**

Drug resistance has been another major obstacle for the treatment effects of both SERMs and AIs. The efficacy and compliance of SERMs or AIs were comprised by both intrinsic (de novo) and acquired drug resistance. In this section, tamoxifen will be used as a representative for SERMs.

#### **1.10.1 Tumor resistance to tamoxifen**

About 30% of ER-positive breast cancer patients do not benefit from tamoxifen because of intrinsic drug resistance. In addition, those ER-positive breast cancer patients who initially respond to tamoxifen treatment may develop acquired drug resistance later. The mechanisms of resistance to tamoxifen may

be attributed to several aspects: 1) cytochrome P450 (CYP) enzymes; 2) loss or altered expressions of ER $\alpha$ / $\beta$ ; 3) phosphorylation of ER $\alpha$ / $\beta$ ; 4) alterations of co-regulatory proteins; 5) growth factor receptor signaling pathways; 6) PI3K/AKT cell survival pathways.

CYPs: since endoxifen is believed to be the most important active metabolite, the drug metabolizing process involved in the production of endoxifen is essential for tamoxifen treatment efficacy. CYP2D6 is the key CYP to metabolize tamoxifen to endoxifen, and single nucleotide polymorphisms (SNPs) of CYP2D6 can result in reduction to null enzyme activity. Also drug-drug interactions between tamoxifen and a CYP2D6 inhibitor will lead to a decreased production of active tamoxifen metabolites, which further leads to decreased treatment efficacy.

Loss or altered expressions of ER $\alpha$ / $\beta$ : since ER- $\alpha$  has been believed to be the most important target of endocrine therapy for ER-positive breast cancer, patients lacking ER- $\alpha$  expression generally do not benefit from tamoxifen treatment. The finding of ER- $\beta$  complicated the estrogen signaling, and several studies have shown that reduced ER- $\beta$  expression may result in resistance to tamoxifen<sup>74</sup>.

Phosphorylation of ER $\alpha$ / $\beta$ : both ER- $\alpha$  and ER- $\beta$  are targets of serine/threonine/tyrosine phosphorylation, among which serine phosphorylation is most prevalent than the other two types. Several studies have shown that serine phosphorylation is more likely to lead to tamoxifen resistance<sup>75</sup>.

Alterations of co-regulatory proteins: as mentioned before, when tamoxifen binds to ERs in the breast tissue, this binding will recruit co-repressors leading to the suppression of estrogen-responsive gene expression. Therefore, the alterations in co-regulatory proteins may result in resistance to tamoxifen. For example, previous studies have shown that reduced expression of a co-repressor NCOR1 is associated with poor tamoxifen clinical response<sup>76</sup>.

Growth factor receptor signaling pathways: many experimental facts suggest that growth factor receptors, such as EGFR, HER2 and IGF-R1 are involved in resistance to tamoxifen. It is believed that EGFR/HER2 expression can lead to resistance to tamoxifen in ER-positive breast cancer, and IGF-IR expression (dependent on ER) is associated tamoxifen resistance<sup>77</sup>.

Overexpression of EGFR or HER2 in ER-positive breast cancer has been associated with drug resistance<sup>78</sup>. It has also been demonstrated that a HER2 inhibitor, gefitinib, can improve the anti-tumor effect of tamoxifen and delay the acquired resistance to tamoxifen<sup>79</sup>. Another study has shown that phosphorylation of IGF-1R is associated with increased resistance to tamoxifen by interacting with EGFR and ERs on membrane.

PI3K/AKT cell survival pathways: PI3K is usually activated in tumor cells by cell surface receptors kinase or G-protein-coupled receptors. PI3K can activate AKT, which further contributes to phosphorylation and cell survival. And, this activation is believed to be able to stimulate ER- $\beta$  transcriptional activity and enhance the recruitment of co-activator<sup>80</sup>. Previous studies have also shown that

increased PI3K-mediated AKT activation can lead to estrogen-independent proliferation and resistance to tamoxifen<sup>81</sup>.

### **1.10.2 Tumor resistance to fulvestrant**

Fulvestrant has fewer drug resistance issues in comparison with tamoxifen or AIs. The mechanism of fulvestrant resistance is usually attributed to growth factor receptor signaling pathways, including EGFR, HER2, MAPK, IGF1R and PI3K/AKT. Previous studies have shown that overexpression of HER2 and its downstream MAPK are associated with resistance to fulvestrant<sup>82</sup>. Other research has shown that overexpression of IGF1R is associated with the resistance to fulvestrant<sup>83</sup>. Very similar to tamoxifen resistance, the increased PI3K-mediated AKT phosphorylation can result in estrogen-independent proliferation and thus resistance to fulvestrant<sup>80</sup>.

### **1.10.3 Tumor resistance to AIs**

Both intrinsic (de novo) and acquired drug resistance to AIs become a major obstacle for the treatment effects of AIs. The mechanisms of AI resistance include: 1) pharmacological mechanisms; 2) alternative sources to estrogen; 3) inherent estrogen insensitivity; 4) activation of hormone signaling pathways; 5) cell survival pathways.

Pharmacological mechanisms: AIs may become ineffective or compromised due to a decreased plasma concentration caused by drug metabolism or drug-drug interactions or both. For example, UDP-

glucuronosyltransferase can deactivate and clear anastrozole and exemestane; therefore, the overexpression of UDP-glucuronosyltransferase may cause resistance to anastrozole and exemestane<sup>84</sup>. In another example, concomitant use of tamoxifen with anastrozole or letrozole will decrease the plasma concentrations of both AIs, which may lead to the ineffectiveness of AI treatment<sup>85,86</sup>.

Alternative sources to estrogen: even though AIs are able to block the synthesis of endogenous estrogen, their effects may be compromised when exogenous estrogenic compounds or estrogen-similar steroids exist<sup>87</sup>. Estrogen-similar steroids, like adrenal androgens, can interact with ERs similarly to estrogen.

Inherent estrogen insensitivity: despite ER-negative breast cancer, the truth is that many ER-positive breast cancers are also resistant to AIs<sup>88</sup>. There are two possible reasons: first, ERs are mutated or aberrant or both; second, co-regulatory proteins are mutated or aberrant or both<sup>89</sup>.

Activation of growth factor receptor signaling pathways: this mechanism of resistance is the same for tamoxifen, fulvestrant and AIs, because the tumor cells become estrogen independent by activating growth factor receptor signaling pathways. As discussed before, this mechanism can be caused by overexpression of EGFR, HER2, MAPK, IGF1R and PI3K/AKT.

Cell survival pathways: there has been a hypothesis that even though AIs can inhibit breast cancer cells growth, effective cell survival is sufficient to maintain overall tumor growth.



### **1.11 Combination of SERMs/SERDs and AIs for the treatment of ER-positive breast cancer**

The hypothesis of combining anti-cancer drugs is that agents with different mechanism of actions and non-overlapping toxicity could be administrated concurrently to maximize anti-tumor effects and alleviate side effects caused by an agent as a monotherapy. An AI is an agent that can suppress estrogen production and a SERM/SERD is an agent that can antagonize estrogenic activity. The combination of a SERM/SERD and an AI is thought to be able to achieve a complete estrogen blockade. To discover synergistic effects of combining a SERM/SERD and an AI, both preclinical studies and clinical trials have been conducted to assess the efficacy and safety profiles of the combination of SERMs/SERDs and AIs. Previous studies have shown that the combination of a SERM/SERD and an AI may improve treatment efficacy and patients' compliance by increasing breast cancer tumor growth inhibition, reducing the risk of drug resistance, and alleviating the side effects.

#### **1.11.1 Increasing treatment efficacy**

Two preclinical studies (fulvestrant + letrozole and fulvestrant + anastrozole in mice models) and one clinical trial (Southwest Oncology Group-226, SWOG-226) have shown the combination of fulvestrant (a SERD) and an AI (letrozole and anastrozole) can increase treatment efficacy. Fulvestrant + letrozole in a mice model: in 2005, Dr. Brodie's group reported a study using

female ovariectomized athymic mice with human ER-positive breast cancer cells transfected with aromatase gene (MCF-7Ca). Mice were assigned to four groups and treated daily with: 1) vehicle (control); 2) fulvestrant alone; 3) letrozole alone; 4) letrozole + fulvestrant. Fulvestrant-treated tumors were unchanged for the first 4 weeks and had doubled their initial volume after 10 weeks of treatment.

Letrozole-treated tumors were inhibited by 40% for the first 8 weeks. However, these tumors slowly returned to their initial size after 17 weeks of treatment and had doubled after 21 weeks of treatment. Combined letrozole/fulvestrant had similar tumor inhibitory ability to letrozole alone in the first 8 weeks, but the combination was able to inhibit tumor growth by 45% after 29 weeks<sup>90</sup>.

Fulvestrant + anastrozole in a mice model: in 2008, Dr. Brodie's group reported another study using the same intratumoral aromatase mice model. Seven treatment arms were tested: 1) anastrozole alone; 2) fulvestrant alone; 3) anastrozole sequential to fulvestrant; 4) fulvestrant sequential to anastrozole; 5) anastrozole + fulvestrant; 6) anastrozole sequential to anastrozole + fulvestrant; 7) fulvestrant sequential to anastrozole + fulvestrant; 8) vehicle (control). The combination of anastrozole and fulvestrant was the most effective treatment arm to inhibit tumor proliferation over time. Also, treatment arms of anastrozole or fulvestrant sequential to anastrozole + fulvestrant were superior to monotherapy anastrozole or fulvestrant or in sequence<sup>91</sup>. SWOG-226 clinical trial: a clinical trial involving 694 post-menopausal women with HR-positive metastatic breast cancer were randomly assigned (1:1 ratio) to receive anastrozole (with crossover to fulvestrant alone) in monotherapy group or the combination of anastrozole and

fulvestrant. The median progression-free survival (PFS) of the combination group was better than the monotherapy group (15.0 months versus 13.5 months, HR=0.80, p-value=0.007). The overall survival (OS) of the combination group was also superior to the monotherapy group (47.7 months versus 41.3 months, HR=0.81, p-value=0.05). Therefore, the conclusion of SWOG-226 is that the combination of anastrozole and fulvestrant was more effective than anastrozole alone or sequential anastrozole and fulvestrant for the treatment of HR-positive metastatic breast cancer. The incidence rate of side effects (greater than or equal to grade 3) were not significantly different between the two groups<sup>92</sup>.

#### **1.11.2 Delaying or halting tumor resistance**

One preclinical study (fulvestrant + anastrozole in mice model) has shown the combination of fulvestrant (a SERD) and an AI (anastrozole) can delay or halt tumor resistance to endocrine therapy. In an intratumoral aromatase mice model described above, anastrozole + fulvestrant from the beginning or in sequence could effectively down-regulate proteins involved in the development of drug resistance, such as insulin-like growth factor type I receptor  $\beta$  (IGF-IR $\beta$ ), mitogen-activated protein kinase (MAPK), p-MAPK, AKT, mammalian target of rapamycin (mTOR), p-mTOR, and ER- $\alpha$ <sup>91</sup>.

#### **1.11.3 Alleviating side effects**

The Anastrozole, Tamoxifen Alone or in Combination (ATAC) trial has shown the combination of tamoxifen (a SERM) and an AI (anastrozole) can

alleviate side effects induced by either tamoxifen or anastrozole. In the ATAC trial, in comparison with anastrozole, the tamoxifen treatment is associated with an increased risk of endometrial cancer (0.5%) and some incidences of new primary cancers (0.2% melanoma); while, in comparison with tamoxifen, the anastrozole treatment is associated with increased risks of side effects including musculoskeletal disorders (27.8%), bone fractures (5.9%) and incidences of new primary cancers (0.8% colorectal cancer and 0.3% lung cancer). In the combination arm (tamoxifen + anastrozole), the rate of endometrial cancer was fewer than the tamoxifen arm (0.3% versus 0.5%), rate of musculoskeletal disorders was less than the anastrozole arm (22.1% versus 27.8%), rate of bone fractures was fewer than the anastrozole arm (4.6% versus 5.9%). Most importantly, the incidence rates of new primary cancers were lowest in the combination arm among the three arms (anastrozole versus tamoxifen versus anastrozole + tamoxifen): colorectal cancer (0.8% versus 0.6% versus 0.3%), lung cancer (0.3% versus 0.2% versus 0.1%), melanoma (0.0% versus 0.2% versus 0.0%) and overall new primary cancer (3.5% versus 3.4% versus 2.6%)<sup>93</sup>. The ATAC trial was designed as a five-year trial with three parallel arms, however, the combination arm (anastrozole + tamoxifen) was discontinued based on the initial analyses at 33 and 47 months of median follow-up. The initial analyses showed no superior therapeutic benefit of the combination arm over the anastrozole monotherapy arm in terms of both disease-free survival (DFS) and breast cancer recurrence<sup>94</sup>. However, there are three facts in the ATAC trial that will challenge a generalized conclusion that all SERM/AI combinations will

inevitably generate no additional benefit in comparison with using an AI or a SERM alone: 1) the pharmacokinetics (PK) of anastrozole was significantly affected by the co-administration of tamoxifen, with a 27% reduction in plasma concentration of anastrozole. Even though plasma concentration of E2 was similar between the combination arm and the anastrozole arm; this cannot lead to a conclusion that higher exposure of anastrozole will not have any contribution to the efficacy; 2) during a long-term treatment of tamoxifen and anastrozole, patients are shown to have resistance towards these medications. Development of resistance to a treatment is important factor that affects long-term treatment efficacy and patients' compliance. With a relatively short period (33 or 47 months), patients may have not developed resistance to anastrozole or tamoxifen yet, and previous studies have shown the potential of combined SERD/AI to delay or halt the development of resistance; 3) this result cannot be generalizable to all SERMs/SERDs and AIs combinations or dual AI/SERM(D) agents.

### **1.12 Research objectives**

Current endocrine therapy for the treatment of ER-positive breast cancer such as SERMs, SERDs and AIs are associated with side effects and drug resistance, which compromise both treatment efficacy and patients' compliance. One possible strategy to improve both treatment outcomes and patients compliance is to develop agents with dual AI and SERM/SERD activities. As shown in previous preclinical studies and clinical trials of combining an AI and a

SERM/SERD, the potential benefits of a dual AI/SERM(D) agent include: 1) increasing treatment efficacy; 2) delaying or halting development of drug resistance; 3) alleviating side effects caused by SERMs, SERDs or AIs. The previous studies by our group have shown that norendoxifen, a metabolite of tamoxifen, is a potent AI *in vitro*, and norendoxifen has high binding affinity for ERs (ER- $\alpha$  and - $\beta$ ) *in vitro*. The potent aromatase inhibitory activity and the high binding affinity for ERs support the idea of further utilizing norendoxifen as a lead compound for the development of dual AI/SERM(D) agents. Moreover, the fact that millions of patients have already been exposed to norendoxifen as a metabolite of tamoxifen supports a low-risk safety profile expected for norendoxifen.

The central hypothesis of this dissertation is that tamoxifen metabolites may possess both aromatase inhibitory and ER antagonistic activity. Studies outlined in this dissertation test this hypothesis through the following specific aims:

Aim 1. To test the inhibitory activity and binding affinities of tamoxifen metabolites against aromatase and ERs (ER- $\alpha$  and - $\beta$ ) *in vitro*.

Aim 2. To test the cellular aromatase inhibitory activity and ERs (ER- $\alpha$  and - $\beta$ ) antagonistic activity by tamoxifen metabolites *in vitro*.

Aim 3. To design, synthesize and characterize norendoxifen analogues with dual aromatase inhibitory activity and ER modulatory activity.

## CHAPTER 2: MATERIALS AND METHODS

### 2.1 Chemical and reagents

The *E*-, mixed (*E,Z*) and *Z*-norendoxifen were provided by Dr. Mark Cushman (Purdue University, West Lafayette, IN). The chemical makeup of the *E*-norendoxifen used in the studies is 100:1 *E,Z*, whereas the *Z*-norendoxifen is 1:10 *E,Z*. They were stored at -20°C without light. Hydroxynorendoxifen was provided by Dr. Mark Cushman (Purdue University, West Lafayette, IN) and it was stored at -20°C without light. Cytochrome P450 (CYP) inhibitor screening kits of aromatase (CYP19), CYP1A2, CYP2A6, CYP3A4, CYP3A5, CYP2C19 and CYP2D6 were purchased from BD Biosciences (San Jose, CA). ERs (ER- $\alpha$  and - $\beta$ ) competitor assay kits were purchased from Invitrogen (Carlsbad, CA). Minimum essential medium (MEM), HPLC-grade methanol, ethyl acetate, sodium hydroxide and sodium chloride were purchased from Fisher Scientific LLC. (Hanover Park, IL). Formic acid, glycine, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 17 $\beta$ -estradiol (E2) and the internal standard (IS), diphenylhydantoin (DPH), were purchased from Sigma-Aldrich (St. Louis, MO). Distilled deionized water was obtained using Nanopure Infinity UV laboratory water system from Barnstead/Thermolyne (Dubuque, IA). Fetal bovine serum (FBS) and charcoal stripped FBS were purchased from Invitrogen (Carlsbad, CA).

## **2.2 Breast cancer cell line**

The cell line used in this study was MCF-7 (Michigan Cancer Foundation-7), a human breast cancer cell line. The MCF-7 cells were provided by Dr. Todd Skaar (Indiana University School of Medicine, Indianapolis, IN). The cells were grown as monolayers in plastic flasks (25 cm<sup>2</sup>) containing MEM with 10% FBS, at 37°C in a humidified 5% CO<sub>2</sub> incubator. The cells were harvested with trypsin. The medium was changed every 72 h.

## **2.3 High-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS)**

### **2.3.1 Stock solutions**

A standard stock solution of hydroxynorendoxifen was prepared by dissolving 6.9 mg of hydroxynorendoxifen in 1837.7 µL of methanol. Two separate stock solutions of hydroxynorendoxifen were prepared and the corresponding concentrations were compared to check the accuracy of the initial stock solution. Sequential dilutions to 0.125, 0.25, 0.5, 1, 5 and 10 ng/mL were made in methanol to prepare standard solutions. A stock solution of DPH, the internal standard, was prepared by dissolving 10 mg of DPH in 10 mL of methanol and the working solution (500 ng/mL) was also prepared by diluting the solution with methanol. Four separate stock solutions of DPH were prepared and the corresponding concentrations were compared to check the accuracy of the initial stock solution. All the stock solutions were prepared on a free-base basis



and all solutions were stored at -20°C. The final biological content of the calibration standards was larger than 98%.

### **2.3.2 Instrumentation and chromatographic conditions, HPLC-MS/MS operation conditions**

Analysis was performed on an API 2000 triple-quadrupole mass spectrometer (Applied Biosystem/MDS Sciex, Foster City, CA) equipped with a turbo ion spray source operating in the positive ionization mode, and controlled by Analyst software Version 1.4.1 in conjunction with Windows 2000. The HPLC system consists of two LC-20AB binary pumps and a SIL-20AHT UFLC auto-sampler with a controller (Shimadzu, Columbia, MD, USA). The chromatographic separation was performed on a phenomenex Luna C<sub>18</sub> column (100 mm × 2.0 mm, 3 µm particle size) together with a phenomenex Luna C<sub>18</sub> guard column (4 mm × 2.0 mm) (Phenomenex, Torrance, CA, USA). The injection volume was 70 µL for each human plasma sample. Before and after each injection, the needle was washed with acetonitrile/H<sub>2</sub>O (75/25, v/v). Mobile phase A consisted of methanol and formic acid (0.05% in water) (55/45, v/v) and mobile phase B consisted of methanol and formic acid (0.05% in water) (90/10, v/v) using the following gradient: linear gradient from 100% mobile phase A to 100% mobile phase B between 0.01 min and 15 minutes, then re-equilibrated to initial conditions (100% mobile phase A) between 15.01 min and 20 min at a consistent flow rate of 0.2 mL/min. Mass spectrometry optimization was achieved by adjusting both the instrument-dependent and compound-dependent parameters

for hydroxynorendoxifen in the positive ionization mode using DPH as an internal standard. The analytes including the internal standard were optimized at a source temperature of 550 °C, under unit resolution for quadrupoles 1 and 3, and were given a dwell time of 100 ms and a setting time of 0 ms. Optimal gas pressures for the analytes including the internal standard were collision gas, 4 psi; curtain gas, 30 psi; ion source gas (1), 30 psi; ion source gas (2), 20 psi; ion spray voltage, 5500 V in the positive mode. Quantification was made using multiple reaction monitoring (MRM) in the positive mode. The MRM transitions of the precursor ions to selected product ions were  $m/z$  376.260  $\rightarrow$  44.026 for hydroxynorendoxifen and was  $m/z$  256  $\rightarrow$  167 for DPH (the internal standard). Additional compound-dependent mass spectrometer parameters are shown in Table 2.

**Table 2. Mass spectrometry settings for hydroxynorendoxifen (Hdn) and the internal standard, diphenylhydantoin (DPH)**

|     | RT<br>(min) | MW<br>(amu) | Q1<br>(amu) | Q3<br>(amu) | DP<br>(V) | EP<br>(V) | CEP<br>(V) | CE<br>(V) | CXP<br>(V) |
|-----|-------------|-------------|-------------|-------------|-----------|-----------|------------|-----------|------------|
| Hdn | 2.24        | 375.5       | 376.3       | 44.03       | 51.0      | 7.0       | 15.0       | 60.0      | 0.0        |
| DPH | 2.35        | 252.3       | 256.0       | 167.0       | 40.0      | 4.0       | 15.0       | 20.0      | 4.0        |

Hdn, hydroxynorendoxifen; DPH, diphenylhydantoin; RT, Retention time; MW, Molecular weight; DP, Declustering potential; EP, Entrance potential; CEP, Cell entrance potential; CE, Collision energy; CXP, Cell exit potential

### 2.3.3 Standard curve

Triplicate standard curves were prepared daily by diluting the hydroxynorendoxifen stock solution using methanol. These dilutions were mixed with drug-free human plasma (total volume of 500 µL) to prepare the standard curves. The triplicate diluted concentrations for standard curves of hydroxynorendoxifen were 0.125, 0.25, 0.5, 1, 5 and 10 ng/mL. Three inter-day standard curves were prepared to test for intra- and inter-day variability.

### 2.3.4 Assay accuracy and precision

Intra- and inter-day accuracy and precision were assessed. Triplicate standard curves were prepared each day (3 independent days) and assayed. The concentration of each hydroxynorendoxifen standard was estimated using the standard curve run on the same day. An average ( $\pm$  SD) of each hydroxynorendoxifen standard was calculated and used to estimate accuracy and precision. Accuracy (%) was calculated by the following equation:  $\text{Accuracy (\%)} = 100\% - 100\% \times (\text{standard concentration} - \text{estimated concentration}) / \text{standard concentration}$ . Precision, expressed as the coefficient of variation (CV), was estimated by the following equation:  $\text{CV (\%)} = 100\% \times (\text{SD of estimated concentrations} / \text{average of estimated concentrations})$  <sup>42</sup>.

### **2.3.5 Extraction efficiency**

Triplicate non-extracted samples containing the same concentrations of hydroxynorendoxifen with the internal standard, DPH, were injected into the HPLC. An average of these samples at each concentration was designated as the 100% extracted sample. Then, triplicate hydroxynorendoxifen samples of each concentration with the internal standard were prepared, extracted and injected into the HPLC. The peak area ratio of hydroxynorendoxifen to the internal standard was compared to that of the non-extracted sample to estimate the extraction efficiency. The average ( $\pm$  SD) extraction efficiency of triplicate samples was reported for each concentration (0.125, 0.25, 0.5, 1, 5 and 10 ng/mL) in standard curves.

### **2.3.6 Extraction procedure for calibration standards**

For calibration standards, 450  $\mu$ L of drug-free human plasma with a known concentration of hydroxynorendoxifen was placed into clean 13-mL screw-cap glass tubes. After adding 25  $\mu$ L of the internal standard solution (500 ng/mL DPH in methanol) into each tube, the mixture was made alkaline by adding 1 mL of 1 M NaOH-glycine buffer (pH=11.3) and vortex-mixed. The 1 M NaOH-glycine buffer (pH=11.3) was prepared by mixing equal volumes of 1 M glycine solution and 1 M NaCl solution. The pH of the buffer was adjusted to 11.3 by adding 1 M NaOH solution. Ethyl acetate (6 mL) was added to each sample. The mixture was mixed on a shaker for 15 minutes and centrifuged for 15 minutes at 3600 g. Then the organic phase was extracted. The organic phase was transferred to a

13 mm × 100 mm glass culture tube and evaporated to dryness. The residue was reconstituted using 160 µL of mobile phase A and a 70 µL aliquot was then injected into the HPLC column.

### **2.3.7 Analysis of patient plasma samples**

Plasma samples from breast cancer patients who had been treated with 20 mg daily oral dosage of tamoxifen for 4 months were analyzed. The patient enrollment criteria, clinical trial design and sampling schedule have been described in detail in a previous publication<sup>95</sup>. Plasma samples of patients analyzed herein were obtained after 4 months of treatment with tamoxifen. The study was approved by the Institutional Review Board of Indiana University and each patient signed informed consent statements before participation. The plasma samples were extracted and processed as described above.

## **2.4 Aromatase inhibition assay**

### **2.4.1 Inhibition of recombinant human CYP isoforms by microsomal incubations**

The activity of each recombinant human CYP isoform was determined by measuring the conversion rate of a fluorometric substrate to its fluorescent metabolite. The activity of aromatase (CYP19) was determined using the metabolism of 7-methoxy-4-trifluoromethylcoumarin (MFC) to 7-hydroxy-4-trifluoromethylcoumarin (HFC). The activities of CYP1A2 and CYP2C19 were determined using the metabolism of 3-cyano-7-ethoxycoumarin (CEC) to 3-cyano-7-hydroxycoumarin (CHC). The activity of CYP2A6 was determined using

the metabolism of coumarin to 7-hydroxycoumarin (HC). The activities of CYP3A4 and CYP3A5 were determined using the metabolism of 7-benzyloxy-4-trifluoromethyl-coumarin (BFC) to 7-hydroxy-4-trifluoromethylcoumarin (HFC). Experimental procedures were essentially as described previously<sup>96</sup>. All of the incubations were performed using incubation times and protein concentrations that were within the linear range for the reaction velocity. All the substrates were dissolved in acetonitrile (25 mM final concentration for MFC, 20 mM final concentration for CEC, 1.1 mM final concentration for coumarin and 50 mM final concentration for BFC). *E*- and mixed norendoxifen were dissolved in methanol/dichloromethane (1:1, v/v, 10 mM final concentration stock). *Z*-Norendoxifen and hydroxynorendoxifen was dissolved in methanol (10 mM final concentration stock). During serial dilutions, all the *E*-, mixed, *Z*-norendoxifen and hydroxynorendoxifen were diluted in methanol to required concentrations. A series of concentrations of *E*-, mixed, *Z*-norendoxifen and hydroxynorendoxifen in a volume of 2  $\mu$ L were mixed with 98  $\mu$ L of NADPH-Cofactor Mix (16.25  $\mu$ M NADP<sup>+</sup>, 825  $\mu$ M MgCl<sub>2</sub>, 825  $\mu$ M glucose-6-phosphate and 0.4 Units/mL glucose-6-phosphate dehydrogenase) and were pre-warmed for 10 minutes at 37°C. The enzyme/substrate mix was prepared with fluorometric substrate, recombinant human CYP isoforms and 0.1 M potassium phosphate buffer (pH 7.4). Reactions were initiated by adding 100  $\mu$ L enzyme/substrate mix to bring the incubation volume to 200  $\mu$ L and incubated for 30 minutes. All the reactions were stopped by adding 75  $\mu$ L of 0.1 M Tris base dissolved in acetonitrile. The amount of fluorescent product was determined immediately by measuring fluorescent

response using a BioTek (Winooski, VT) Synergy 2 fluorometric plate reader. Excitation-emission wavelengths for MFC metabolite and BFC metabolite were 409-530 nm, for CEC metabolite were 410-460 nm, for coumarin metabolite were 390-460 nm. Standard curves were constructed using the appropriate fluorescent metabolite standards. Quantification of samples was performed by applying the linear regression equation of the standard curve to the fluorescence response. The limits of quantification for the metabolites of MFC, CEC, coumarin and BFC were 24.7 pmol, 66.7 pmol, 74.1 pmol and 222.2 pmol in a final volume of 200  $\mu$ L, respectively, with intra- and inter-assay coefficients of variations less than 10%.

#### **2.4.2 Kinetic analysis of recombinant human CYP isoforms**

The rates of metabolite formation in the presence of test inhibitors were compared with those in the control incubation, in which the inhibitor was replaced with vehicle. The extent of enzyme inhibition was expressed as a percentage of remaining enzyme activity compared to the control. The  $IC_{50}$  was determined as the inhibitor concentration which brought about a 50% reduction in enzyme activity by fitting all the data to a one-site competition equation using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA). To characterize the inhibitory mechanism of norendoxifen against aromatase (CYP19), CYP1A2, CYP2A6, CYP3A4, CYP3A5, CYP2C19 and CYP2D6, all inhibitory data of norendoxifen at different substrate concentrations were plotted as Lineweaver-Burk, Eadie-Hofstee and Dixon plots. The inhibitory constant  $K_i$  values were

determined by nonlinear least square regression analysis using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA). Before modeling the data using nonlinear models, initial information about the inhibitory mechanism was obtained by visual inspection of Lineweaver-Burk, Eadie-Hofstee and Dixon plots. Final decisions on the mechanism of inhibition were made on model-derived parameters, such as  $R^2$  (or R Square) and absolute sum of squares.

## **2.5 Estrogen receptor binding assay**

The binding affinity for estrogen receptor- $\alpha$  and - $\beta$  (ER- $\alpha$  and ER- $\beta$ ) was determined by measuring the change of polarization value when the fluorescent estrogen ligand, ES2, was displaced by test compounds. Experimental procedures were essentially as described previously<sup>48</sup>. The fluorescent estrogen ligand, ES2, was provided in methanol/water (4:1, v/v) with a concentration of 1800 nM. Recombinant human ERs (ER- $\alpha$  and ER- $\beta$ ) were provided in buffer (50 mM bis-tris propane, 400 mM KCl, 2 mM DTT, 1 mM EDTA, and 10% glycerol), with the concentration of 734 nM and 3800 nM, respectively. The test compounds, including *E*-, mixed, *Z*-norendoxifen and hydroxynorendoxifen, were dissolved in methanol. The sample solutions (1  $\mu$ L) were mixed well with 49  $\mu$ L of ES2 screening buffer (100 mM potassium phosphate, 100  $\mu$ g/mL BGG, and 0.02% NaN<sub>3</sub>). The ER- $\alpha$ /ES2 complex was prepared with the fluorescent estrogen ligand ES2, human recombinant ER- $\alpha$ , and ES2 screening buffer with the concentration of 9 nM ES2 and 30 nM ER- $\alpha$ . The ER- $\beta$ /ES2 complex was prepared with the fluorescent estrogen ligand ES2, human recombinant ER- $\beta$ ,



and ES2 screening buffer with the concentration of 9 nM ES2 and 20 nM ER- $\beta$ . Reactions were initiated by adding 50  $\mu$ L of ER/ES2 complex to bring the incubation volume to 100  $\mu$ L and incubated for 2 h avoiding light. The polarization value was determined by measuring fluorescent response using a BioTek (Winooski, VT) Synergy 2 fluorometric plate reader. Excitation–emission wavelengths for fluorescence polarization were 485–530 nm. The polarization value in the presence of the test competitors were compared with those in control in which the competitor was replaced with vehicle. The extent of competition was expressed as a percentage of the remaining polarization compared to the control. EC<sub>50</sub> values were determined as the competitor concentrations which brought about half reduction in polarization value by fitting all the data to a one-site competition equation using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA).

## **2.6 Tritiated water assay**

Aromatase activity in MCF-7 cells was measured using the tritiated water release assay, based on the formation of tritiated water from the aromatization of a labeled androgenic substrate 1 $\beta$ -<sup>3</sup>H(N)-androst-4-ene-3,17-dione (NET-926; PerkinElmer, Boston, MA)<sup>97,98</sup>. MCF-7 cells are seeded into a 12-well plate at 500,000 cells per well with MEM supplemented with 10% FBS. When a homogeneous monolayer of preconfluent MCF-7 cells was reached on days 48 h of the experiment, media were aspirated and replaced by FBS free media for 30 minutes incubation. Various concentrations of norendoxifen and

hydroxynorendoxifen were dissolved in methanol and the final concentration of methanol was 0.1%. Then the FBS free media was aspired and the MCF-7 cells were treated with various concentrations of norendoxifen or hydroxynorendoxifen (12 concentrations on a logarithmic scale from 0.56 nM to 100000 nM) in 1 mL FBS free media containing 40 nM  $1\beta$ - $^3\text{H}$ (N)-androst-4-ene-3,17-dione with a specific activity of 25.5 Ci/mmol and vehicle (0.1% methanol). After 4 h incubation at 37°C in an atmosphere containing 5% CO<sub>2</sub>, the reaction mixture was removed and extracted with two volumes of chloroform to terminate the reaction and to extract the unused substrate and steroids. After vortexing the solution for 15 minutes, the supernatant was centrifuged at 3000 g for 15 minutes. Then the resulting aqueous supernatant was absorbed with an equal volume of 10% dextran-coated charcoal and vortexed for 15 minutes to eliminate the residual steroids. After 15 minutes centrifugation at 3000 g, the aqueous phase was added into 10 mL scintillation cocktail. Incubations in the absence of cells were included as negative controls, the amount of radioactivity in tritiated water [ $^3\text{H}$ ] was corrected by subtracting the blank values from each sample<sup>99</sup>.

## **2.7 Gene expression analysis using real-time polymerase chain reaction (PCR)**

### **2.7.1 Ribonucleic acid (RNA) extraction and concentration measurement**

MCF-7 cells were seeded at a density of 5000 cells/well in 96-well plates with MEM containing 10% FBS and allowed to attach for 24 h. Before experimental treatments, these cells were preconditioned in MEM containing

10% charcoal stripped FBS for 3 days to remove the estrogens. Then the MCF-7 cells treated with test compounds or experimental controls for 24 h were harvested for progesterone receptor (PGR) messenger ribonucleic acid (mRNA) extraction. Before ribonucleic acid (RNA) extraction, genomic DNA was eliminated. RNA was extracted from approximately  $3 \times 10^5$  cells by RNeasy Plus Mini Kit (Qiagen Inc., Valencia, California, USA). The RNA concentration was measured using the Qubit RNA BR assay (Life Technologies Corp., Carlsbad, CA) for the Qubit 2.0 fluorometer (Life Technologies Corp., Carlsbad, CA). The RNA was stored at  $-80^{\circ}\text{C}$  before further use.

### **2.7.2 Complementary deoxyribonucleic acid (cDNA) synthesis**

Complementary deoxyribonucleic acid (cDNA) for the real-time quantitative polymerase chain reaction (PCR) assay was synthesized from DNase-treated total RNA using the QuantiTect reverse transcription kit (Qiagen Inc., Valencia, California, USA).

### **2.7.3 Real-time quantitative polymerase chain reaction (PCR) for cDNA**

The cDNA was amplified with TaqMan Universal PCR Master Mix (Applied Biosystems Inc., Carlsbad, CA), then PCR was performed in the QuantStudio 12K Flex Real-Time PCR System (Life Technologies Corp., Carlsbad, CA). Progesterone receptor (PGR, FAM, Hs01556702, Life Technologies Corp., Carlsbad, CA) gene was the target gene, while glyceraldehyde-3-phosphate dehydrogenase (GAPDH, VIC, Hs02758991, Life Technologies Corp., Carlsbad,

CA) gene expression was quantified to normalize each sample. A total of 40 amplification cycles were performed. Quantitative values of amplification were obtained from the threshold cycle ( $C_t$ ) defined as the cycle number at which the fluorescent signal is first recorded above the background and is determined during the exponential phase of PCR rather than at the endpoint. The  $2^{-\Delta\Delta C_t}$  method was used to determine the relative mRNA expression, and the results were expressed as percentages of antagonistic effects compared to E2-stimulated PGR mRNA expression (considered as 100%). If amplification was not seen within 40 cycles, the measured RNA was considered to be undetected.

## **2.8 Cell proliferation assay**

The anti-proliferation effect of norendoxifen and hydroxynorendoxifen on breast cancer cells (MCF-7 cells) was tested using MTT cell proliferation assay. MCF-7 cells were seeded at a density of 5000 cells/well in 96-well plates with MEM containing 10% FBS and allowed to attach for 24 h. Before the experimental treatments, these cells were preconditioned in MEM containing 10% charcoal stripped FBS for 3 days to remove the estrogens. Various concentrations of norendoxifen and hydroxynorendoxifen were dissolved in methanol and the final concentration of methanol was less than 0.1%. The MCF-7 cells were treated with various concentrations of norendoxifen or hydroxynorendoxifen (8 concentrations on a logarithmic scale from 0.15 nM to 2500 nM) in the presence of 1 nM  $\beta$ -estradiol or vehicle (0.1% methanol). Treatment with 1 nM  $\beta$ -estradiol alone was used as positive controls and

treatment with endoxifen was used as negative controls for 48 h<sup>39</sup>. Following these treatments, the MCF-7 cells were treated with 20  $\mu$ L of 5 mg/mL MTT solution dissolved in PBS and incubated at 37°C for 4 h to allow MTT metabolization. The medium containing MTT was then removed and the precipitated crystals were dissolved by adding 150  $\mu$ L/well acidic isopropyl alcohol (0.04 N HCl). Absorbance was measured at 540 nm using a BioTek (Winooski, VT) Synergy 2 fluorometric plate reader. Each experiment was carried out with three independent experiments. Efficiency of test compounds was assessed by plotting cell viability versus test compound concentration (on a log scale) into competition equation using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA). The IG<sub>50</sub> value is expressed as mean standard deviation of three determinations, in which each concentration group contains six replicates. Data were analyzed using student's t-test with statistical significance of  $P < 0.05$ .

## **2.9 Subjects**

Eligible pre- and post-menopausal women (older than 18 years old) with diagnosed breast cancer were recruited from the Simon Cancer Center at Indiana University School of Medicine, from Lombardi Comprehensive Cancer Center at Georgetown University Medical Center and from the Breast Oncology Program at University of Michigan Comprehensive Cancer Center. Women were excluded from the study if they had received cytotoxic chemotherapy or adjuvant radiation therapy or if they had taken any hormonal therapy other than tamoxifen. These patients were permitted to take vitamin E, selective serotonin reuptake

inhibitors (SSRIs) or herbal remedies; however, they were excluded if they were receiving chronic corticosteroid therapy or using clonidine, bellergal or megestrol acetate for the treatment of hot flashes. Women were also excluded if they were pregnant or lactating. The study protocol was approved by the institutional review boards of all the three participating study sites. All the enrolled patients provided written informed consent before study entry.

## **2.10 Trial Design**

All the patients were administrated with Tamoxifen orally 20 mg per day for 12 months and were followed up on an outpatient basis at 1, 4, 8, and 12 months after the start of tamoxifen therapy. The patient's blood samples (5 mL) were collected in heparinized tubes and the plasma of blood collection was separated with 1 hour using centrifugation at 2060 g. All the plasma and whole blood samples were transferred to cryogenic vials (Corning, Cambridge, MA) and shipped to the Division of Clinical Pharmacology at Indiana University School of Medicine on dry ice and were stored at -80°C. We picked up 10 plasma samples randomly from the initial 4 months of tamoxifen therapy to measure the plasma concentration of hydroxynorendoxifen. This time period was selected because tamoxifen serum concentrations have already reached steady state by 4 months and the confounding effects of concomitant medication usage and premature trial discontinuation would be limited for more possibility.

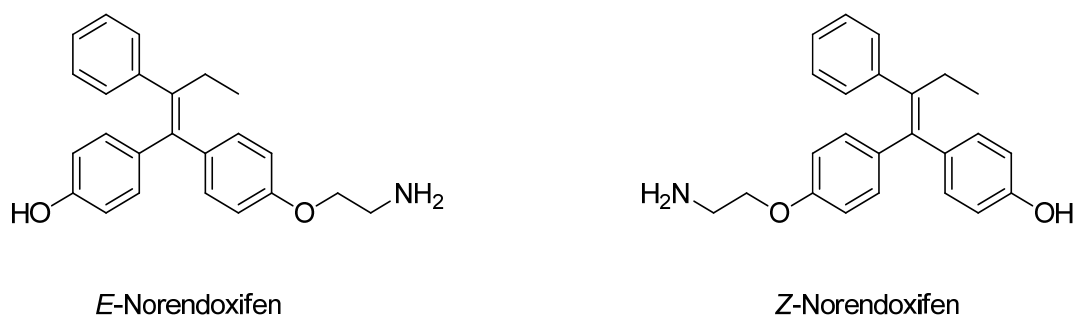
## CHAPTER 3: AROMATASE INHIBITORY ACTIVITY AND ESTROGEN RECEPTOR BINDING AFFINITY OF TAMOXIFEN METABOLITES *IN* *VITRO*

### 3.1 Introduction

Previous studies by our group have shown that norendoxifen can inhibit recombinant aromatase (CYP19) via a competitive mechanism with an  $IC_{50}$  of 90 nM. Further studies have shown that norendoxifen can bind to ER- $\alpha$  and - $\beta$  with  $EC_{50}$  values of  $26.9 \pm 4.8$  nM and  $35.2 \pm 16.8$  nM, respectively<sup>48</sup>.

Aromatase is a member of cytochrome P450 (CYP) superfamily, which consists aromatase (CYP19), CYP1A2, CYP2A6, CYP3A4, CYP3A5, CYP2B6, CYP2C9, CYP2C19 and CYP2D6. The selectivity of inhibitory ability of norendoxifen against CYP1A2, CYP2A6, CYP3A4 and CYP3A5 has not been described, and it follows that further studies are required to determine the effects of norendoxifen on these CYPs. This is important to further develop norendoxifen as a drug or a lead compound. The double bond in norendoxifen leads to *E*- and *Z*-isomers, which may possess different inhibitory activities against CYPs due to their different chemical structures.

Our collaborating chemical group in Purdue University headed by Dr. Mark Cushman has succeeded in synthesizing the *E*- and *Z*-isomers of norendoxifen<sup>48</sup>. The chemical structures of *E*- and *Z*-isomers of norendoxifen are shown in Figure 2.



**Figure 2. Chemical structures of *E*- and *Z*- norendoxifen**

Hydroxynorendoxifen (*N,N*-didesmethyl-4,4'-dihydroxytamoxifen) is a derivative of norendoxifen, which is under preclinical development as dual SERM/AI agent for the treatment of estrogen-related conditions. Aromatase (CYP19), CYP1A2, CYP2A6, CYP3A4, CYP3A5 and CYP2D6 are important components of CYP superfamily, so further studies are required to determine the potency and specificity of hydroxynorendoxifen on aromatase inhibition.

In this chapter, we will focus on **Aim 1** to test the inhibitory activity and binding affinity of tamoxifen metabolites against aromatase and ERs (ER- $\alpha$  and - $\beta$ ) *in vitro*. Studies outlined in **Aim 1** test this hypothesis through the following specific sub-aims:

**Aim 1A.** To identify a novel tamoxifen metabolite in patients taking tamoxifen.

**Aim 1B.** To test the potency and selectivity of aromatase inhibition by norendoxifen and hydroxynorendoxifen *in vitro*.



**Aim 1C.** To characterize ER binding affinity of norendoxifen and hydroxynorendoxifen *in vitro*.

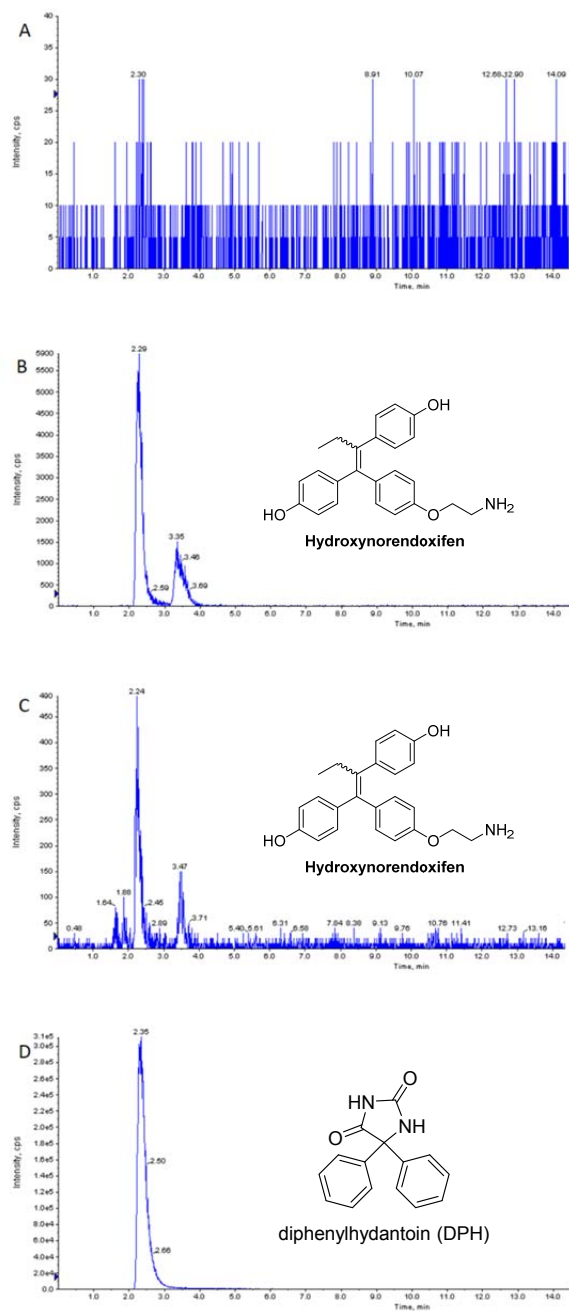
## **3.2 Results**

### **3.2.1 Hydroxynorendoxifen as a novel tamoxifen metabolite in patients taking tamoxifen**

#### **3.2.1.1 Chromatography**

Hydroxynorendoxifen exhibited good solubility in methanol, but was less soluble in water. Starting from mobile phase A containing 55% methanol to mobile phase B containing 100% methanol, hydroxynorendoxifen showed good chromatography with baseline resolution. Figure 3 shows representative chromatograms of extracted drug-free human plasma (Figure 3A), a human plasma standard containing 10 ng/mL hydroxynorendoxifen (Figure 3B), a human plasma sample obtained from a subject after oral administration of 20 mg tamoxifen per day for 4 months (Figure 3C) and a human plasma standard containing 500 ng/mL DPH as internal standard (Figure 3D), respectively. The retention times for hydroxynorendoxifen and the internal standard, DPH, were 2.24 min and 2.35 min, respectively. Comparison of drug-free human plasma (Figure 3A) with human plasma spiked with hydroxynorendoxifen (Figure 3B), or the internal standard (Figure 3D), indicated that there is no endogenous source of interference at or near the retention times of hydroxynorendoxifen or the internal standard. Similarly, no interfering peaks were observed in the chromatograms of patient plasma samples (which were obtained during the

administration of a 20 mg daily oral dose of tamoxifen for 4 months). DPH was a suitable internal standard for this assay because it was stable, with a relatively short retention time and appropriate peak shape.



**Figure 3. Representative chromatograms of extracted samples**

(A) drug-free human plasma (B) human plasma standard containing 10 ng/mL hydroxynorendoxifen (C) human plasma obtained from a subject after administration of a 20 mg daily oral dose of tamoxifen for 4 months (D) human plasma standard containing 500 ng/mL internal standard, diphenylhydantoin (DPH).

#### **3.2.1.2 Linearity**

The standard curves for hydroxynorendoxifen were constructed by injecting standard concentrations of hydroxynorendoxifen into the HPLC-MS/MS. The linear regression calibration curves based on seven points were constructed for hydroxynorendoxifen by plotting the peak area ratio of hydroxynorendoxifen to the internal standard, DPH, versus the concentrations of human plasma hydroxynorendoxifen standards. A typical linear regression equation for hydroxynorendoxifen included a slope of 0.002 and a y-intercept of 0.000. The calibration curves were consistently linear from 0.125 to 10 ng/mL for hydroxynorendoxifen.

#### **3.2.1.3 Extraction efficiency**

The extraction efficiency of hydroxynorendoxifen from human plasma was obtained by comparing the extracted standards to non-extracted standards. The extraction efficiency of hydroxynorendoxifen was greater than 86% at each concentration with a mean value ( $\pm$  SD)  $91.0 \pm 2.7\%$  (Table 3). The high

extraction efficiency indicates there was little to no degradation of hydroxynorendoxifen during the extraction procedure.

**Table 3. Extraction efficiency (%) of hydroxynorendoxifen in human plasma**

| <b>Hydroxynorendoxifen (ng/mL)</b> | <b>Extraction efficiency (%)</b> |
|------------------------------------|----------------------------------|
| <b>0.125</b>                       | <b>93.8 ± 6.9</b>                |
| <b>0.25</b>                        | <b>91.5 ± 2.5</b>                |
| <b>0.5</b>                         | <b>93.9 ± 3.8</b>                |
| <b>1</b>                           | <b>90.7 ± 2.6</b>                |
| <b>5</b>                           | <b>89.2 ± 8.9</b>                |
| <b>10</b>                          | <b>86.9 ± 6.0</b>                |
| <b>Average ± SD</b>                | <b>91.0 ± 2.7</b>                |

#### **3.2.1.4 Assay accuracy and precision**

Triplicate standards in human plasma were extracted and analyzed to assess the intra-day variability of this method. Accuracy and precision (coefficient of variations, CVs) of hydroxynorendoxifen throughout the standard curves are shown in Table 4. The results showed that the intra-day accuracy ranged from 94.2% to 107.7% and the inter-day accuracy ranged from 99.2 to 103.3%. Intra-day CVs were less than 10% (range: 0.9-9.9%) for all the hydroxynorendoxifen standards over the range of concentrations tested (from 0.125 to 10 ng/mL).

Human plasma samples containing 0.125-10 ng/mL of hydroxynorendoxifen were extracted and analyzed daily to determine inter-day variability. As shown in Table 4, inter-day CVs were also less than 10% (range: 1.4-9.5%) for all the hydroxynorendoxifen standards over a range of concentrations (from 0.125 to 10 ng/mL).

**Table 4. Intra-day and inter-day accuracy and precision (coefficient of variations, CVs) of hydroxynorendoxifen**

| Theoretical Concentration (ng/mL) | Intra-day               |              |         | Inter-day               |              |         |
|-----------------------------------|-------------------------|--------------|---------|-------------------------|--------------|---------|
|                                   | Estimated conc. (ng/mL) | Accuracy (%) | CVs (%) | Estimated conc. (ng/mL) | Accuracy (%) | CVs (%) |
| 0.125                             | 0.13 ± 0.01             | 107.0        | 7.9     | 0.13 ± 0.01             | 102.9        | 6.4     |
| 0.25                              | 0.26 ± 0.02             | 104.1        | 8.8     | 0.26 ± 0.01             | 103.3        | 1.2     |
| 0.5                               | 0.54 ± 0.01             | 107.7        | 2.7     | 0.50 ± 0.04             | 100.8        | 8.4     |
| 1                                 | 0.94 ± 0.09             | 94.2         | 9.9     | 1.00 ± 0.09             | 99.7         | 9.5     |
| 5                                 | 5.09 ± 0.16             | 101.8        | 3.0     | 5.02 ± 0.24             | 100.3        | 4.8     |
| 10                                | 10.00 ± 0.09            | 100.0        | 0.9     | 9.92 ± 0.14             | 99.2         | 1.4     |

### ***3.2.1.5 Limit of blank (LOB), limit of detection (LOD) and lower limit of quantification (LLOQ)***

The limit of blank (LOB) and limit of detection (LOD) for this assay method were determined using the standard method described in guideline EP17 of the Clinical and Laboratory Standards Institute (CLSI)<sup>100</sup>. The limit of blank (LOB) is

defined as the highest apparent analyte concentration expected to be found, when replicates of drug-free human plasma (blank samples) are tested. LOB is estimated by calculating the mean value and the standard deviation of blank samples ( $SD_{\text{blank}}$ ), which is according to  $LOB = \text{mean}_{\text{blank}} + 1.645 \times (SD_{\text{blank}})$ . For this assay method, the LOB was 0.0092 ng/mL. The limit of detection (LOD) is the lowest analyte concentration likely to be reliably distinguished from the LOB and at which concentration the detection is feasible. LOD was determined by utilizing both LOB and triplicates of 0.125 ng/mL hydroxynorendoxifen standards (the low concentration standard samples), which is according to  $LOD = LOB + 1.645 \times (SD_{\text{low concentration samples}})$ . For this assay method, the LOD was 0.0202 ng/mL. When the lowest standard concentration was 0.125 ng/mL, the signal-to-noise ratio was 5.5. The intra- and inter-day CVs for hydroxynorendoxifen at the concentration of 0.125 ng/mL were 7.88% and 6.37%, respectively. Therefore, concentrations of hydroxynorendoxifen in human plasma larger than 0.125 ng/mL can not only be efficiently detected, they can also be protected from bias and imprecision using this method. Since the observed bias and imprecision at the lowest sample concentration (0.125 ng/mL) meet the requirements for total error for hydroxynorendoxifen with acceptable accuracy and precision, the lower limit of quantification (LLOQ) for this assay method was the lowest calibration standard concentration of hydroxynorendoxifen, which is 0.125 ng/mL. The linearity obtained and the LLOQ determined (0.125 ng/mL) are appropriate to allow successful measurement of

human plasma concentrations of hydroxynorendoxifen, the metabolite of tamoxifen, in a prospective clinical trial of tamoxifen administration.

#### ***3.2.1.6 Stability of hydroxynorendoxifen in methanol***

A solution of 10 µM hydroxynorendoxifen in methanol was frozen at -20°C and then thawed and assayed on 3 different days within one week. Stability of hydroxynorendoxifen stock solution after being stored at -20°C for 3 months and its stability in the auto sampler over 24 h pending to analysis were evaluated. All the CVs were less than 10%, and no change was observed with regard to the ratio of hydroxynorendoxifen to the internal standard, DPH, due to storage.

#### ***3.2.1.7 Pharmacokinetic study (plasma concentration of hydroxynorendoxifen)***

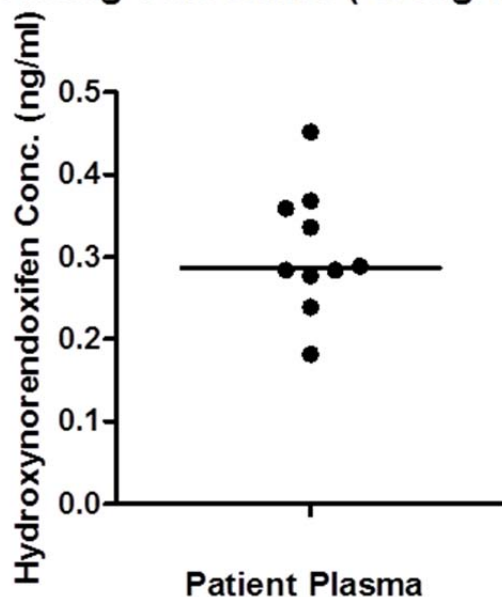
The freeze-thaw plasma was determined over two cycles within 5 days, which shows no change regarding the peak area ratio of hydroxynorendoxifen to DPH (internal standard). This method was applied to a clinical study of 10 women who were taking tamoxifen orally (20 mg/day) for the treatment of breast cancer for one year. The plasma concentrations of hydroxynorendoxifen, the novel metabolite of tamoxifen, quantified in these patients at 4 months are illustrated in Table 5 and Figure 4.

**Table 5. Plasma concentrations of hydroxynorendoxifen in 10 women after oral administration of 20 mg tamoxifen per day for 4 months**

| <b>Sample id</b>    | <b>Concentration of<br/>hydroxynorendoxifen<br/>(ng/mL)</b> | <b>Concentration of<br/>hydroxynorendoxifen<br/>(nM)</b> |
|---------------------|---|--|
| <b>1809-035-04</b>  | <b>0.289</b>  | <b>0.770</b>   |
| <b>1809-032-04</b>  | <b>0.368</b>  | <b>0.980</b>   |
| <b>1809-030-04</b>  | <b>0.284</b>  | <b>0.756</b>   |
| <b>1809-010-04</b>  | <b>0.182</b>  | <b>0.485</b>   |
| <b>1809-028-04</b>  | <b>0.277</b>  | <b>0.738</b>   |
| <b>1809-024-04</b>  | <b>0.284</b>  | <b>0.756</b>   |
| <b>1809-022-04</b>  | <b>0.452</b>  | <b>1.204</b>   |
| <b>1809-027-04</b>  | <b>0.336</b>  | <b>0.895</b>   |
| <b>1809-006-04</b>  | <b>0.359</b>  | <b>0.956</b>   |
| <b>1809-042-04</b>  | <b>0.239</b>  | <b>0.637</b>   |
| <b>Average ± SD</b> | <b>0.307 ± 0.075</b>  | <b>0.817 ± 0.200</b>                                     |



**Plasma Collected from 10 Patients  
Taking Tamoxifen (20 mg/day)**



**Figure 4. Plasma concentrations of hydroxynorendoxifen in 10 breast cancer patients plasma samples**

These samples were collected from 10 patients, who took oral administration of 20 mg/day tamoxifen for the treatment of breast cancer. The cross bar represents the median concentration of hydroxynorendoxifen in this patients plasma.

**3.2.2 Potency and selectivity of aromatase inhibition by norendoxifen and hydroxynorendoxifen *in vitro***

The  $IC_{50}$  and  $K_i$  values of important tamoxifen metabolites against aromatase (CYP19) were shown in Table 6. Norendoxifen (*E,Z*) can inhibit aromatase with a  $K_i$  value of  $70 \pm 9$  nM. Hydroxynorendoxifen (*E,Z*) was a more

potent aromatase inhibitor (AI) with a  $K_i$  value of  $20 \pm 4$  nM. *N*-DMT and endoxifen were very weak AIs with  $K_i$  values larger than 1000 nM, while, 4-HT and tamoxifen itself did not show any aromatase inhibitory activity.

**Table 6.  $IC_{50}$  and  $K_i$  values of important tamoxifen metabolites against aromatase (CYP19)**

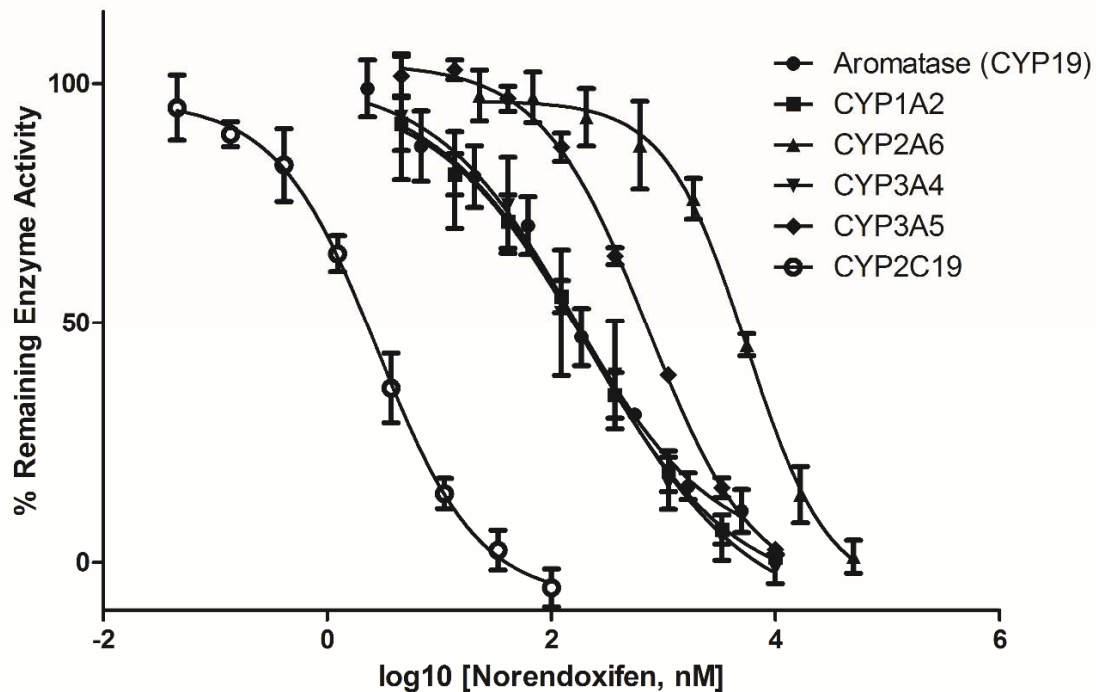
|                            | <b><math>IC_{50}</math> (nM) against<br/>aromatase (CYP19)</b> | <b><math>K_i</math> (nM) against<br/>aromatase (CYP19)</b> |
|----------------------------|--|--|
| <b>Tamoxifen</b>           | <b>N.A.</b>  | <b>N.A.</b>  |
| <b><i>N</i>-DMT</b>        | <b>20700</b>   | <b>15900</b>   |
| <b>4-HT</b>                | <b>N.A.</b>  | <b>N.A.</b>  |
| <b>Endoxifen</b>           | <b>6100</b>  | <b>4000</b>  |
| <b>Norendoxifen</b>        | <b><math>131 \pm 54</math></b>                                 | <b><math>70 \pm 9</math></b>                               |
| <b>Hydroxynorendoxifen</b> | <b><math>45 \pm 3</math></b>                                   | <b><math>20 \pm 4</math></b>                               |

N.A. = Not Active

To test the selectivity of norendoxifen (*E,Z*) against aromatase (CYP19), the inhibition of CYPs by norendoxifen, including aromatase (CYP19), CYP1A2, CYP2A6, CYP3A4, CYP3A5 and CYP2C19, were tested using microsomal incubations. Figure 5 showed the inhibitory potency of norendoxifen (*E,Z*) against CYP enzymes. Norendoxifen can inhibit recombinant CYP2C19, aromatase

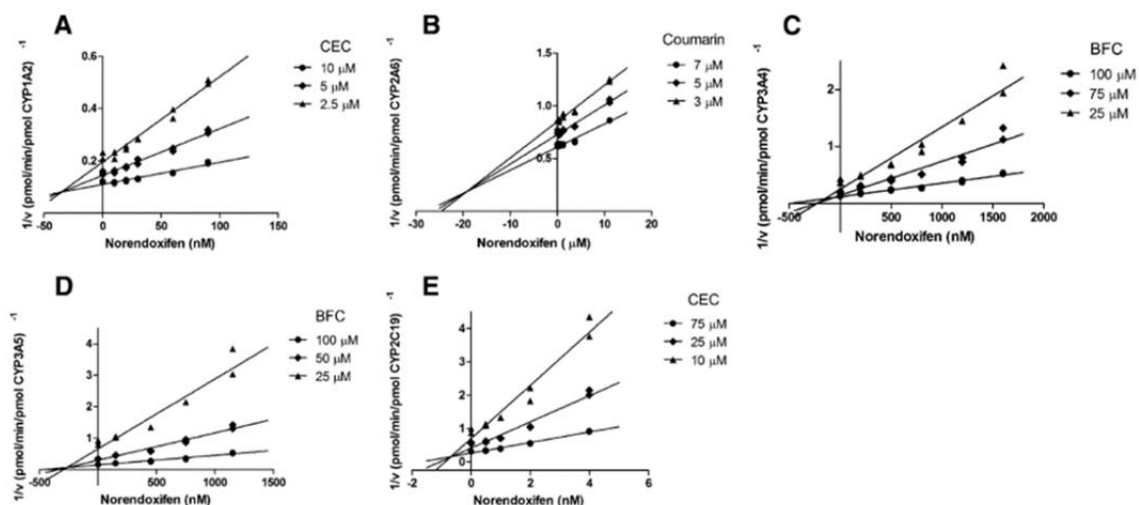
(CYP19), CYP1A2, CYP3A4, CYP3A5 and CYP2A6 with  $K_i$  values of  $2.80 \pm 0.29$  nM,  $131 \pm 54$  nM,  $207 \pm 26$  nM,  $285 \pm 81$  nM,  $723 \pm 27$  nM and  $6373 \pm 983$  nM, respectively. Norendoxifen showed significantly different inhibitory activity against CYP2C19 when CEC was used as a substrate, compared to that seen when R-omeprazole was used. In the previous studies by our group, the inhibition of CYP2C19 activity by norendoxifen in HLMs using R-omeprazole as a substrate was very weak, with <25% enzyme activity reduced when the concentration of norendoxifen was 5  $\mu$ M. Our findings indicated that norendoxifen may have substrate-dependent inhibition, and further study on the inhibition of CYP2C19 by tamoxifen metabolites should be conducted.

Figure 6 shows the dixon plots of inhibition of CYP1A2, CYP2A6, CYP3A4, CYP3A5 and CYP2C19 by norendoxifen (*E,Z*). Norendoxifen inhibited aromatase (CYP19), CYP1A2, CYP2A6 and CYP2C19 via a competitive mechanism, while norendoxifen inhibited CYP3A4 and CYP3A5 via a noncompetitive mechanism.



**Figure 5. Inhibition of cytochrome P450 enzymes by norendoxifen (*E,Z*).**

In the presence of a range of concentrations of norendoxifen (*E,Z*), the remaining enzyme activity of recombinant aromatase (CYP19) (●), CYP1A2 (■), CYP2A6 (▲), CYP3A4 (▼), CYP3A5 (◆) and CYP2C19 (○) were determined by measuring the conversion rates from specific fluorometric substrates to their fluorescent metabolites. The extent of enzyme inhibition was expressed as percentage of remaining enzyme activity compared to the control. Each point represents the mean of four independent incubations and error bars represent the standard deviations of 4 independent points.

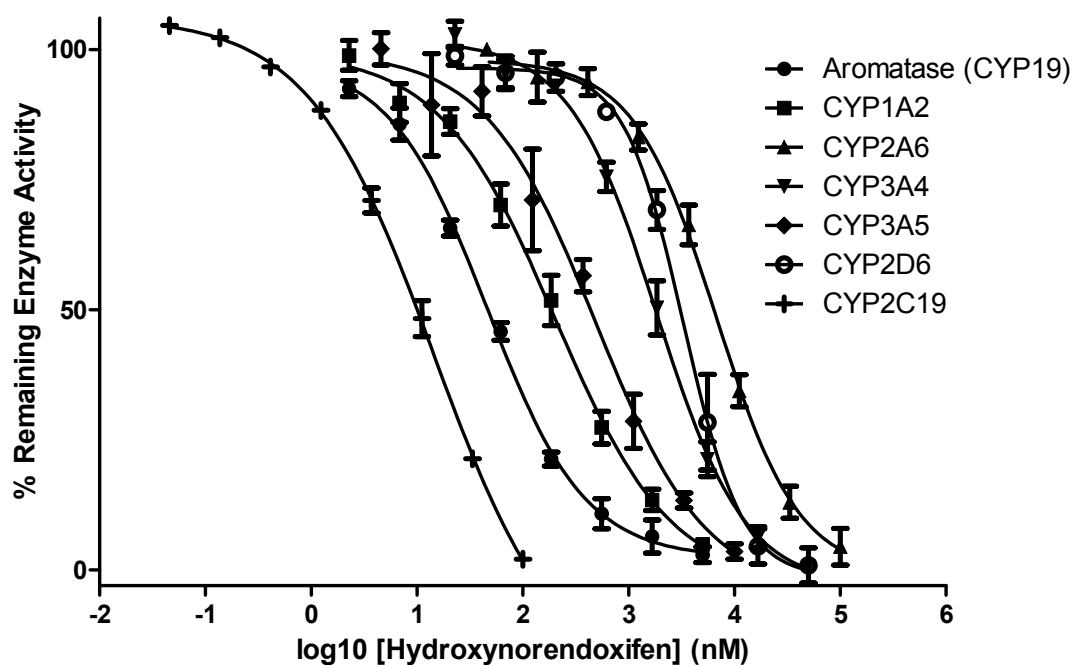


**Figure 6. Dixon plots of inhibition of CYP1A2, CYP2A6, CYP3A4, CYP3A5 and CYP2C19 by norendoxifen (*E,Z*)**

(A) CEC (2.5 to 10  $\mu\text{M}$ ) was incubated with 2.5 nM recombinant CYP1A2 in the presence of increasing norendoxifen concentrations (0 to 90 nM). (B) Coumarin (3 to 7  $\mu\text{M}$ ) was incubated with 5 nM recombinant CYP2A6 in the presence of increasing norendoxifen concentrations (0 to 11  $\mu\text{M}$ ). (C) BFC (25 to 100  $\mu\text{M}$ ) was incubated with 5 nM recombinant CYP3A4 in the presence of increasing norendoxifen concentrations (0 to 1600 nM). (D) BFC (25 to 100  $\mu\text{M}$ ) was incubated with 5 nM recombinant CYP3A5 in the presence of increasing norendoxifen concentrations (0 to 1150 nM). (E) CEC (10 to 75  $\mu\text{M}$ ) was incubated with 5 nM recombinant CYP2C19 in the presence of increasing norendoxifen concentrations (0 to 4 nM). Each point represents one incubation, and two independent incubations were conducted for each concentration of norendoxifen and substrate.

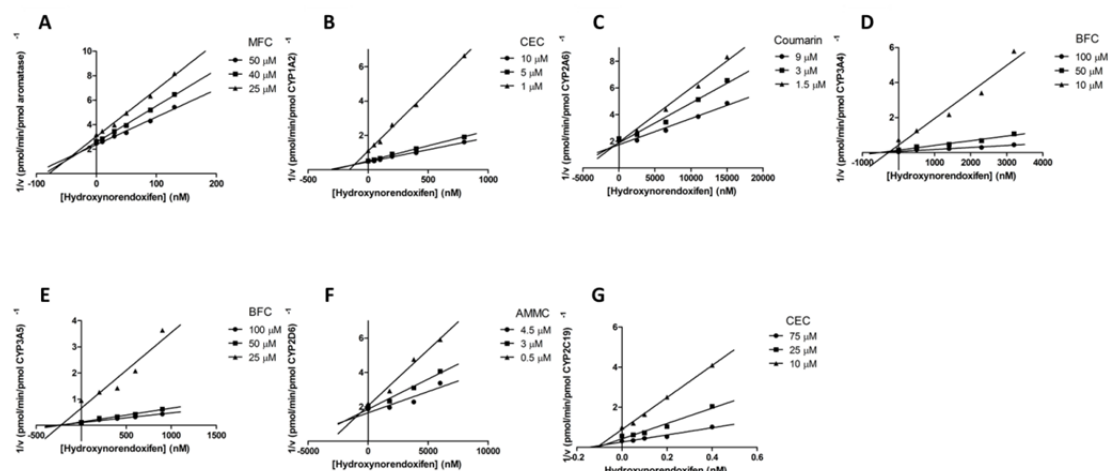
Similarly, the inhibition of CYPs by hydroxynorendoxifen (*E,Z*) was tested using microsomal incubations to determine the inhibitory specificity of hydroxynorendoxifen against aromatase (CYP19) and understand whether the inhibitory ability extends to other important CYPs. Figure 7 showed the inhibitory activity of hydroxynorendoxifen against human recombinant CYP2C19, aromatase (CYP19), CYP1A2, CYP2A6, CYP3A4, CYP3A5 and CYP2D6. Hydroxynorendoxifen is able to inhibit these recombinant CYPs with  $IC_{50}$  values of  $2.10 \pm 0.35$  nM,  $45 \pm 3$  nM,  $207 \pm 56$  nM,  $6730 \pm 659$  nM,  $1884 \pm 336$  nM,  $556 \pm 52$  nM and  $3597 \pm 395$  nM, respectively.

Figure 8 shows the dixon plots of inhibition of these enzymes by hydroxynorendoxifen (*E,Z*). Hydroxynorendoxifen inhibits aromatase (CYP19), CYP1A2, CYP2A6 and CYP2D6 via a competitive mechanism with  $K_i$  values of  $20 \pm 4$  nM,  $56 \pm 5$  nM,  $710 \pm 212$  nM and  $423 \pm 124$  nM, respectively. Hydroxynorendoxifen inhibits CYP3A4 and CYP3A5 via a noncompetitive mechanism with  $K_i$  values of  $1635 \pm 89$  nM and  $855 \pm 14$  nM, respectively.



**Figure 7. Inhibition of cytochrome P450 enzymes by hydroxynorendoxifen (*E,Z*)**

In the presence of a range of concentrations of hydroxynorendoxifen (*E,Z*), the remaining enzyme activity of recombinant aromatase (CYP19) (●), CYP1A2 (■), CYP2A6 (▲), CYP3A4 (▼), CYP3A5 (◆), CYP2D6 (○) and CYP2C19 (+) were determined by measuring the conversion rates from specific fluorometric substrates to their fluorescent metabolites. The extent of enzyme inhibition was expressed as a percentage of remaining enzyme activity compared to the control. Each point represents the mean of four independent incubations and error bars represent the standard deviations of four independent determinations.



**Figure 8. Dixon plots of inhibition of aromatase (CYP19), CYP1A2, CYP2A6, CYP3A4, CYP3A5, CYP2D6 and CYP2C19 by hydroxynorendoxifen (E,Z)**

(A) MFC (25 to 50  $\mu\text{M}$ ) was incubated with 7.5 nM recombinant aromatase (CYP19) in the presence of increasing hydroxynorendoxifen concentrations (0 to 130 nM). (B) CEC (1 to 10  $\mu\text{M}$ ) was incubated with 2.5 nM recombinant CYP1A2 in the presence of increasing hydroxynorendoxifen concentrations (0 to 800 nM). (C) Coumarin (1.5 to 9  $\mu\text{M}$ ) was incubated with 5 nM recombinant CYP2A6 in the presence of increasing hydroxynorendoxifen concentrations (0 to 15  $\mu\text{M}$ ). (D) BFC (10 to 100  $\mu\text{M}$ ) was incubated with 5 nM recombinant CYP3A4 in the presence of increasing hydroxynorendoxifen concentrations (0 to 3200 nM). (E) BFC (25 to 100  $\mu\text{M}$ ) was incubated with 5 nM recombinant CYP3A5 in the presence of increasing hydroxynorendoxifen concentrations (0 to 900 nM). (F) AMMC (0.5 to 4.5  $\mu\text{M}$ ) was incubated with 7.5 nM recombinant CYP2D6 in the presence of increasing hydroxynorendoxifen concentrations (0 to 6  $\mu\text{M}$ ). (G) CEC (10 to 75  $\mu\text{M}$ ) was incubated with 5 nM recombinant CYP2C19 in the presence of



increasing hydroxynorendoxifen concentrations (0 to 4 nM). Each point represents the mean of two independent incubations.

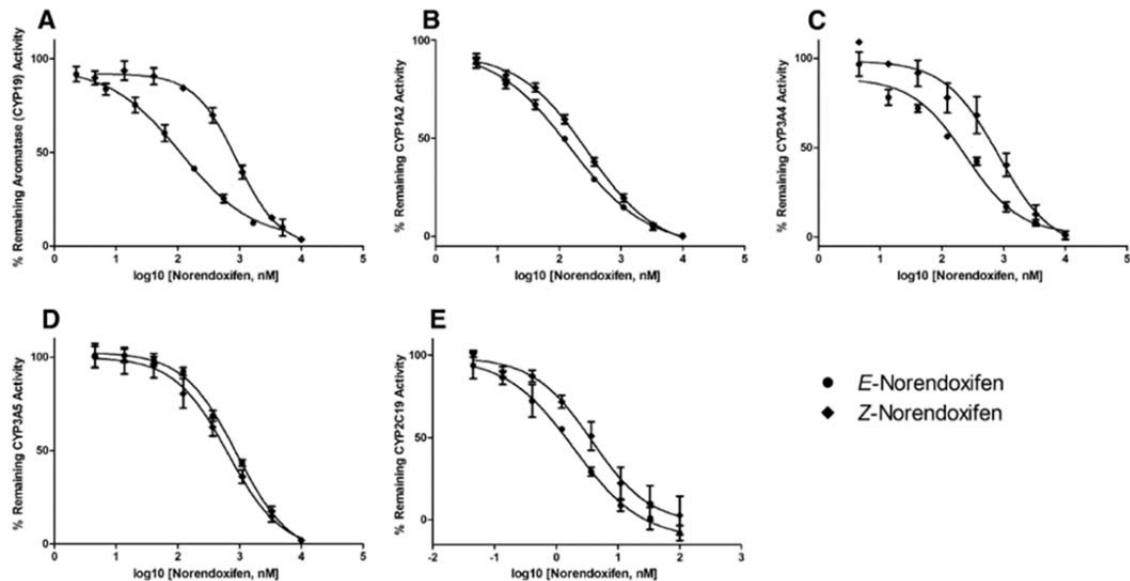
IC<sub>50</sub> and K<sub>i</sub> values of norendoxifen (E,Z) and hydroxynorendoxifen (E,Z) against important CYPs are shown in Table 7.

**Table 7. IC<sub>50</sub> and K<sub>i</sub> values of norendoxifen (E,Z) and hydroxynorendoxifen (E,Z) against important cytochrome P450 enzymes**

| Important CYPs                | Norendoxifen          |                     |                      | Hydroxynorendoxifen   |                     |                      |
|-------------------------------|-----------------------|---------------------|----------------------|-----------------------|---------------------|----------------------|
|                               | IC <sub>50</sub> (nM) | K <sub>i</sub> (nM) | Inhibitory mechanism | IC <sub>50</sub> (nM) | K <sub>i</sub> (nM) | Inhibitory mechanism |
| Recombinant CYP19 (aromatase) | 131 ± 54              | 70 ± 9              | Competitive          | 45 ± 3                | 20 ± 4              | Competitive          |
| Recombinant CYP1A2            | 207 ± 26              | 76 ± 3              | Competitive          | 207 ± 56              | 56 ± 5              | Competitive          |
| Recombinant CYP2A6            | 6373 ± 983            | 2176 ± 256          | Competitive          | 6730 ± 659            | 710 ± 212           | Competitive          |
| Recombinant CYP3A4            | 285 ± 81              | 375 ± 6             | Non-competitive      | 1884 ± 336            | 1635 ± 89           | Non-competitive      |
| Recombinant CYP3A5            | 723 ± 27              | 829 ± 62            | Non-competitive      | 556 ± 52              | 855 ± 14            | Non-competitive      |
| Recombinant CYP2C19           | 2.80 ± 0.29           | 0.56 ± 0.02         | Competitive          | 2.10 ± 0.35           | 0.64 ± 0.11         | Competitive          |
| Recombinant CYP2D6            | N.A.                  | N.A.                | N.A.                 | 3597 ± 395            | 423 ± 124           | Competitive          |
| Recombinant CYP2B6            | N.A.                  | N.A.                | N.A.                 | N.A.                  | N.A.                | N.A.                 |

CYPs, cytochrome P450 enzymes

To explore and determine the isomers' selective effects of norendoxifen against CYPs, the inhibitory activities of *E*- and *Z*-norendoxifen against human recombinant aromatase (CYP19), CYP1A2, CYP3A4, CYP3A5 and CYP2C19 were further tested in the same way using microsomal incubations (Table 8, Figure 9). The IC<sub>50</sub> values of *E*- and *Z*-norendoxifen against recombinant aromatase (CYP19) were 98 ± 40 nM and 1053 ± 185 nM, respectively (Figure 9A). While the IC<sub>50</sub> values of *E*- and *Z*-norendoxifen against recombinant CYP1A2 were 160 ± 22 nM and 285 ± 43 nM, respectively (Figure 9B). The IC<sub>50</sub> values of *E*- and *Z*-norendoxifen against recombinant CYP3A4 were 182 ± 79 nM and 925 ± 145 nM, respectively (Figure 9C). The IC<sub>50</sub> values of *E*- and *Z*-norendoxifen against recombinant CYP3A5 were 930 ± 66 nM and 655 ± 27 nM, respectively (Figure 9D). The IC<sub>50</sub> values of *E*- and *Z*-norendoxifen against recombinant CYP2C19 were 1.90 ± 0.35 nM and 3.88 ± 0.79 nM, respectively (Figure 9E).



**Figure 9. Inhibition of aromatase (CYP19), CYP1A2, CYP3A4, CYP3A5 and CYP2C19 by *E*- and *Z*-norendoxifen.**

(A) The remaining enzyme activity of recombinant aromatase (CYP19) in the presence of a range of concentrations of *E*-norendoxifen (●) and *Z*-norendoxifen (◆) were determined by measuring the formation rates from MFC to HFC. (B) The remaining enzyme activity of recombinant CYP1A2 in the presence of a range of concentrations of *E*-norendoxifen (●) and *Z*-norendoxifen (◆) were determined by measuring the formation rates from CEC to CHC. (C) The remaining enzyme activity of recombinant CYP3A4 in the presence of a range of concentrations of *E*-norendoxifen (●) and *Z*-norendoxifen (◆) were determined by measuring the formation rates from BFC to HFC. (D) The remaining enzyme activity of recombinant CYP3A5 in the presence of a range of concentrations of *E*-norendoxifen (●) and *Z*-norendoxifen (◆) were determined by measuring the formation rates from BFC to HFC. (E) The remaining enzyme activity of

recombinant CYP2C19 in the presence of a range of concentrations of *E*-norendoxifen (●) and *Z*-norendoxifen (◆) were determined by measuring the formation rates from CEC to CHC. Each point represents the mean of four independent incubations and error bars represent the standard deviations of four independent points.

**Table 8. IC<sub>50</sub> and K<sub>i</sub> values of *E*- and *Z*-norendoxifen against important cytochrome P450 enzymes**

| CYPs                          | IC <sub>50</sub>       |                        | K <sub>i</sub>         |                        |
|-------------------------------|------------------------|------------------------|------------------------|------------------------|
|                               | <i>E</i> -Norendoxifen | <i>Z</i> -Norendoxifen | <i>E</i> -Norendoxifen | <i>Z</i> -Norendoxifen |
| Recombinant CYP19 (aromatase) | 98 ± 40                | 1053 ± 185             | 48 ± 3                 | 445 ± 6                |
| Recombinant CYP1A2            | 160 ± 22               | 285 ± 43               | 49 ± 3                 | 96 ± 8                 |
| Recombinant CYP3A4            | 182 ± 79               | 925 ± 145              | 242 ± 9                | 910 ± 59               |
| Recombinant CYP3A5            | 930 ± 66               | 655 ± 27               | 859 ± 76               | 707 ± 17               |
| Recombinant CYP2C19           | 1.90 ± 0.35            | 3.88 ± 0.79            | 0.48 ± 0.05            | 0.70 ± 0.11            |

CYPs, cytochrome P450 enzymes

### 3.2.3 ER binding affinity of norendoxifen (*E,Z*) and hydroxynorendoxifen (*E,Z*) *in vitro*

The EC<sub>50</sub> values of important tamoxifen metabolites to ERs (ER-α and ER-β) are shown in Table 9. Norendoxifen (*E,Z*) can bind to ERs with EC<sub>50</sub> values of 27 ± 5 nM and 35 ± 17 nM, respectively. Hydroxynorendoxifen (*E,Z*) has higher binding affinity with EC<sub>50</sub> values of 19 ± 8 nM and 11 ± 2 nM, respectively.

**Table 9. EC<sub>50</sub> values of important tamoxifen metabolites to ERs (ER-α and ER-β)**

|                            | Binding affinity (EC <sub>50</sub> , nM) for ER-α | Binding affinity (EC <sub>50</sub> , nM) for ER-β |
|----------------------------|---|---|
| <b>Tamoxifen</b>           | <b>60.9</b>                                       | <b>188</b>  |
| <b><i>N</i>-DMT</b>        | <b>N.A.</b>                                       | <b>N.A.</b>                                       |
| <b>4-HT</b>                | <b>29.6</b>                                       | <b>26.1</b>                                       |
| <b>Endoxifen</b>           | <b>28.2</b>                                       | <b>30.5</b>                                       |
| <b>Norendoxifen</b>        | <b>27 ± 5</b>                                     | <b>35 ± 17</b>                                    |
| <b>Hydroxynorendoxifen</b> | <b>19 ± 8</b>                                     | <b>11 ± 2</b>                                     |

N.A. = Not Active

### 3.3 Discussion

Results summary for chapter 3 (aromatase inhibitory activity and estrogen receptor binding affinity of tamoxifen metabolites *in vitro*):

- 1) Hydroxynorendoxifen is a novel metabolite of tamoxifen, with an average plasma concentration of 0.82 nM (0.31 ng/mL) in patients taking 20 mg/day tamoxifen after 4 months.
- 2) Norendoxifen and hydroxynorendoxifen are potent aromatase inhibitors (AIs), with  $K_i$  values of 70 nM and 20 nM, respectively.
- 3) Norendoxifen and hydroxynorendoxifen are relatively selective for aromatase inhibition. The order of inhibitory potency of norendoxifen against important CYPs were human recombinant CYP2C19 > aromatase (CYP19) > CYP1A2 > CYP3A4 > CYP3A5 > CYP2A6. The order of inhibitory potency of hydroxynorendoxifen against important CYPs were human recombinant CYP2C19 > aromatase (CYP19) > CYP1A2 > CYP2D6 > CYP2A6 > CYP3A5 > CYP3A4.
- 4) Norendoxifen and hydroxynorendoxifen have high binding affinity against ER- $\alpha$  and ER- $\beta$ , with  $EC_{50}$  values less than 35 nM. In comparison with 4-HT or endoxifen, norendoxifen had similar binding affinity to ERs and hydroxynorendoxifen had even higher binding affinity to ERs *in vitro*.
- 5) Norendoxifen has high isomer selectivity against human recombinant aromatase (CYP19). *E*-Norendoxifen had 9.3-fold higher inhibitory ability than *Z*-norendoxifen against aromatase (CYP19). *E*-Norendoxifen

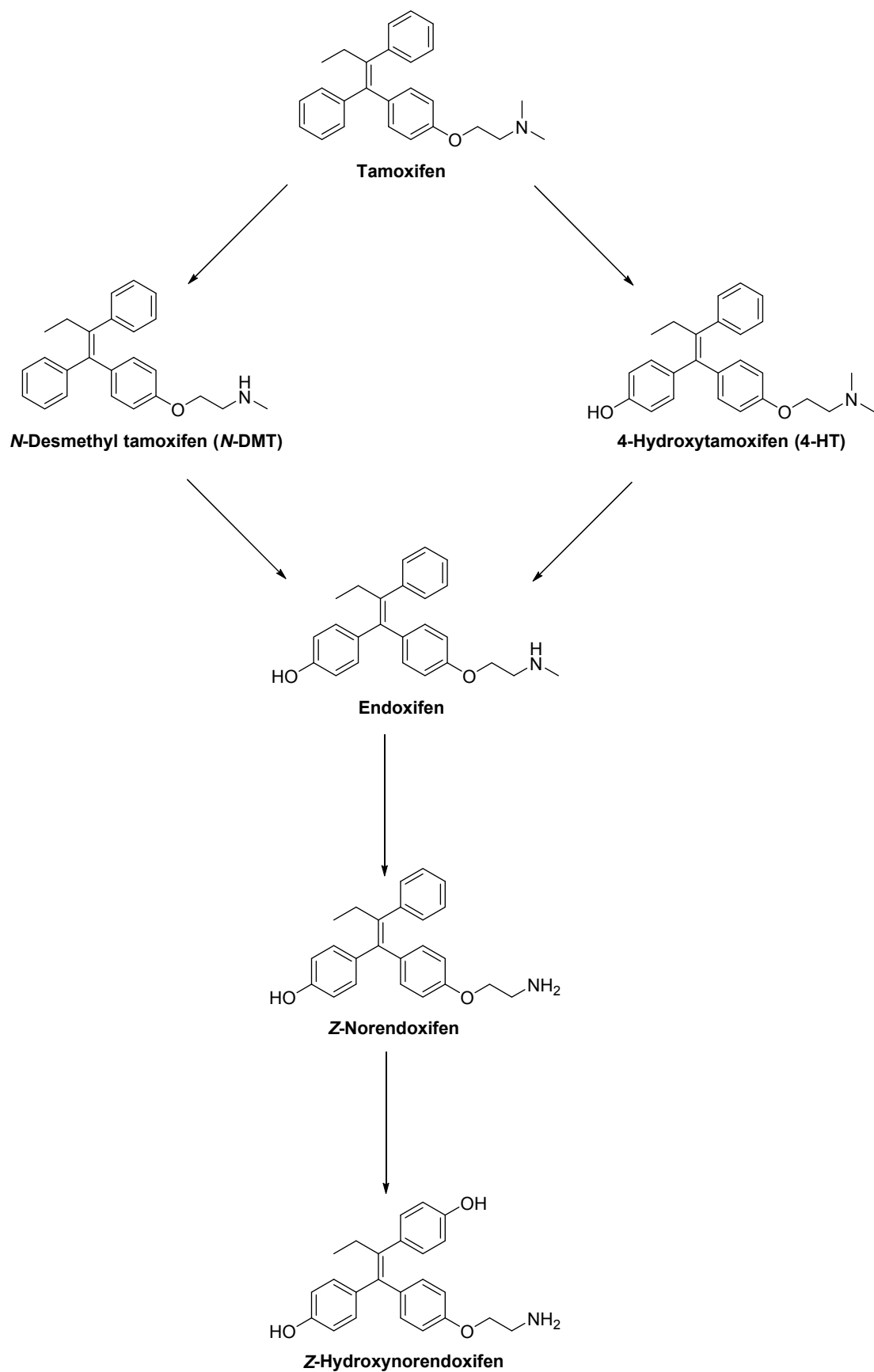
inhibited CYP1A2 and CYP3A4 2.0-fold and 3.7-fold, respectively, more potently than *Z*-norendoxifen.

As hydroxynorendoxifen was identified as a novel metabolite of tamoxifen, HPLC-MS/MS method will be required to characterize hydroxynorendoxifen during both preclinical and clinical pharmacokinetic (PK) studies. The method presented here describes a sensitive and reproducible human PK assay to identify and quantify hydroxynorendoxifen, a novel metabolite of tamoxifen, using HPLC-MS/MS. The method should make it possible to conduct detailed PK and PD studies of tamoxifen and hydroxynorendoxifen, which will allow better understanding of the contribution of hydroxynorendoxifen to the anti-cancer activity of tamoxifen and will allow further development of this dual SERM/AI agent, an important step towards development of this class of compounds as clinical treatments for breast cancer and estrogen-related conditions.

Tamoxifen is extensively metabolized in humans, involving both active and inactive metabolites via various oxidative and conjugated routes, in which cytochrome P450 enzymes played dominant roles in the metabolism of tamoxifen<sup>101</sup>. It has been believed that the clinical response of tamoxifen for the treatment of breast cancer results from a series of effects from its different active metabolites, which contribute to high binding affinity to the ER and agonist/antagonist profiles<sup>102</sup>. The metabolism of tamoxifen results in two main metabolites, *N*-desmethyl tamoxifen (*N*-DMT) and 4-hydroxytamoxifen (4-HT) via CYP3A4 and CYP2D6, respectively. Endoxifen is mainly produced via CYP2D6-mediated hydroxylation of *N*-DMT and via CYP3A4-mediated *N*-demethylation of

4-HT<sup>103</sup>. Among the tamoxifen metabolites, 4-HT and endoxifen have been shown to bind to ERs with similar affinity and have ER antagonistic activity<sup>40</sup>. However, since the mean steady-state plasma concentration of endoxifen is 5 to 7 times higher than that of 4-HT, endoxifen is believed to be the most important active metabolite of tamoxifen as an anti-breast cancer pro-drug<sup>104</sup>. Studies have shown that the tissue concentrations of endoxifen are higher, appearing to be 10-100 times more than plasma concentrations of endoxifen<sup>105</sup>. *N*-DMT and endoxifen can inhibit human recombinant aromatase (CYP19) with IC<sub>50</sub> values of 6.1  $\mu$ M and 20.7  $\mu$ M *in vitro*, respectively<sup>106</sup>. Figure 10 describes the possible metabolic pathway involved in the production of hydroxynorendoxifen from tamoxifen based on *in vitro* studies.





**Figure 10. Possible metabolic pathway involved in the production of hydroxynorendoxifen from tamoxifen**

The metabolism of tamoxifen results in two main metabolites, *N*-desmethyl-tamoxifen (*N*-DMT) and 4-hydroxytamoxifen (4-HT). Both *N*-DMT and 4-HT are converted into endoxifen, a minor metabolite. Endoxifen is demethylated to norendoxifen, after which hydroxynorendoxifen is formed by hydroxylation of norendoxifen.

In this work, aromatase inhibitory activity of hydroxynorendoxifen was tested using microsomal incubations. Hydroxynorendoxifen has more potent aromatase inhibitory activity than norendoxifen *in vitro* with a  $K_i$  value of  $20 \pm 4$  nM. As shown in Table 10, the ratios of tamoxifen metabolites and their corresponding  $K_i$  values ( $[I]/K_i$ ) were calculated. Typically, when  $[I]/K_i$  is larger than 0.1, the inhibitory effects would be considered significant. Our data resulted in  $[I]/K_i$  values of 0.03, 0.04 and 0.04 for *N*-DMT, norendoxifen and hydroxynorendoxifen, respectively. Even though these values did not reach the 0.1 criteria, previous studies have shown that the tissue concentrations of tamoxifen metabolites are higher, appearing to be 10-100 times more than plasma concentrations of tamoxifen metabolites. Therefore, the potent aromatase inhibitory activity of norendoxifen or hydroxynorendoxifen should not be ignored due to the potential higher level of these metabolites produced by higher tissue tamoxifen concentrations. These results emphasize that the

contributions of aromatase inhibitory activity of *N*-DMT and endoxifen should be also considered to be clinical effects of tamoxifen.

**Table 10. Average plasma concentrations of tamoxifen metabolites divided by corresponding  $K_i$  values against aromatase (CYP19)**

|                            | <b>Average plasma concentration (nM)</b> | <b>Average <math>K_i</math> (nM) against aromatase (CYP19)</b> | <b>[I]/<math>K_i</math> (average plasma concentration/<math>K_i</math>)</b> |
|----------------------------|--|--|---|
| <b>Tamoxifen</b>           | <b>372.5</b>                             | <b>N.A.</b>  | <b>N.A.</b>   |
| <b><i>N</i>-DMT</b>        | <b>653.4</b>                             | <b>20700</b>   | <b>0.03</b>   |
| <b>4-HT</b>                | <b>9.5</b>                               | <b>N.A.</b>  | <b>N.A.</b>   |
| <b>Endoxifen</b>           | <b>78.0</b>                              | <b>6100</b>  | <b>0.01</b>   |
| <b>Norendoxifen</b>        | <b>3</b>                                 | <b>70</b>  | <b>0.04</b>   |
| <b>Hydroxynorendoxifen</b> | <b>0.817</b>                             | <b>20</b>  | <b>0.04</b>   |

N.A. = Not Active

Previous studies in our group have characterized the inhibitory ability of norendoxifen against human recombinant aromatase (CYP19), CYP2B6, CYP2D6, human liver CYP2C9 and CYP2C19. Other members of the CYP superfamily, including CYP1A2, CYP2A6, CYP3A4 and CYP3A5, also play important roles. In human liver, CYP1A2 accounts for 13% of the CYP content and catalyzes the primary metabolic route for a number of important drugs,

including caffeine, clozapine, flutamide, lidocaine, olanzapine and zolmitriptan<sup>25</sup>.

CYP2A6 is a major CYP in human liver, specifically involved in the oxidative metabolism of nicotine. It is also involved in the metabolism of pharmaceutical agents such as methoxyflurane, halothane, losigamone, letrozole, valproic acid, disulfiram and fadrozole<sup>26,27</sup>. CYP3A4 and CYP3A5 account for about 50% of the CYP content and are the predominant CYP contributors to metabolism in human liver, accounting for 40-60% of the oxidative metabolism of marketed drugs<sup>28</sup>.

Any proposed new drug that undergoes significant metabolism by CYP superfamily should be evaluated for CYPs inhibition and further for drug-drug interactions (DDIs). In this work, we tested inhibition of important CYPs by norendoxifen and hydroxynorendoxifen. The results show that both norendoxifen and hydroxynorendoxifen are relatively selective for aromatase (CYP19) inhibition.

Since both norendoxifen and hydroxynorendoxifen share similar chemical structures with endoxifen, it is possible that they can function as SERMs. In this context, the binding affinity of norendoxifen and hydroxynorendoxifen for ERs was conducted using microsomal incubations. In comparison with 4-HT or endoxifen, norendoxifen had similar binding affinity for ERs and hydroxynorendoxifen had even higher binding affinity for ERs *in vitro*.

As minor metabolites of tamoxifen, norendoxifen and hydroxynorendoxifen turn out to be the most potent AIs and possess the highest binding affinity for ERs among all the known metabolites that we have tested *in vitro*. Our results reveal a complex metabolism of tamoxifen that may lead to some metabolites

that are able to act as AIs or act as SERMs or dual SERM/AI agents. Since the concentration of norendoxifen and hydroxynorendoxifen in the tissues may be higher, as demonstrated with endoxifen, it may significantly increase the clinical activity of tamoxifen *in vivo*, due to its potent inhibitory ability against CYP19 and high binding affinity for ERs.

To explore and determine the isomer selective effects of norendoxifen against CYP enzymes, studies were conducted on testing the inhibitory activity of *E*- and *Z*-norendoxifen against human recombinant aromatase (CYP19), CYP1A2, CYP3A4, CYP3A5 and CYP2C19. Norendoxifen has high isomer selectivity against aromatase (CYP19). *E*-Norendoxifen had 9.3-fold higher inhibitory ability than *Z*-Norendoxifen against aromatase (CYP19). *E*-Norendoxifen inhibited CYP1A2 and CYP3A4 2.0-fold and 3.7-fold, respectively, more potently than *Z*-norendoxifen. *E*- and *Z*-norendoxifen had similar inhibitory ability against CYP3A5 and CYP2C19. The double bond of norendoxifen does have a significant impact on the inhibitory activities of its *E*- and *Z*-isomers against aromatase (CYP19). The high selectivity of *E*-norendoxifen also provides new information for the development of potent AIs. While the main form of norendoxifen as the metabolite of tamoxifen in human body is the *Z*-isomer, the toxicity and metabolism of the *E*-norendoxifen deserve more study.

## CHAPTER 4: CELLULAR AROMATASE INHIBITORY ACTIVITY AND ESTROGEN RECEPTOR ANTAGONISTIC ABILITY OF NORENDOXIFEN AND HYDROXYNORENDOXIFEN

### 4.1 Introduction

Norendoxifen and hydroxynorendoxifen have been considered new classes of SERMs or AIs or dual SERM/AI agents under development, therefore, they should be evaluated for cellular aromatase inhibitory activity, ER-responsive gene expression antagonistic activity and anti-proliferation activity against breast cancer cells. These assays would become strong assets to characterize aromatase inhibitory activity and antagonistic activity of both norendoxifen and hydroxynorendoxifen against breast cancer cells.

In this chapter, we will focus on **Aim 2** to test the cellular aromatase inhibitory activity and antagonistic activity against ERs (ER- $\alpha$  and - $\beta$ ) by tamoxifen metabolites *in vitro*. Studies outlined in **Aim 2** test this hypothesis through the following specific sub-aims:

**Aim 2A.** To test the cellular aromatase inhibitory activity by norendoxifen and hydroxynorendoxifen.

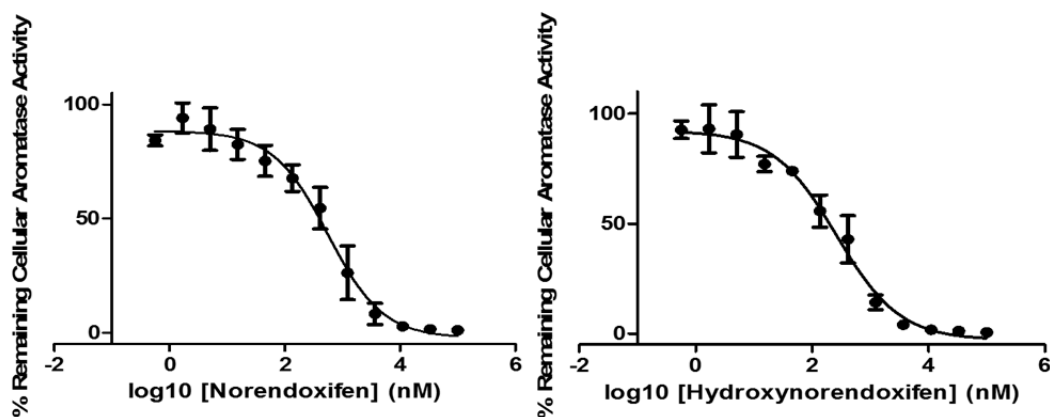
**Aim 2B.** To test activity of norendoxifen and hydroxynorendoxifen to antagonize ER-responsive gene expression (in estradiol-stimulated breast cancer cells).

**Aim 2C.** To test anti-proliferation activity of norendoxifen and hydroxynorendoxifen against breast cancer cells (stimulated by estradiol).

## 4.2 Results

### 4.2.1 Cellular aromatase activity inhibition by norendoxifen and hydroxynorendoxifen

As shown in Figure 11, norendoxifen (*E,Z*) and hydroxynorendoxifen (*E,Z*) can inhibit cellular aromatase activity in MCF-7 cells with  $IC_{50}$  values of  $565 \pm 111$  nM and  $282 \pm 68$  nM, respectively.



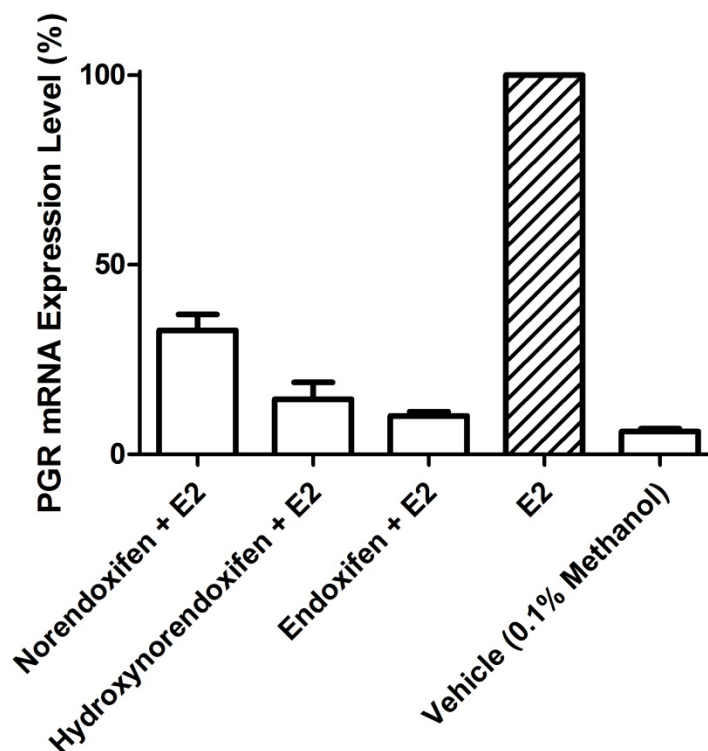
**Figure 11. Inhibition of cellular aromatase activity in MCF-7 cells by norendoxifen (*E,Z*) and hydroxynorendoxifen (*E,Z*)**

In the presence of a range of concentrations of norendoxifen (*E,Z*) and hydroxynorendoxifen (*E,Z*), the remaining aromatase activity in MCF-7 cells were determined using the tritiated water assay. The extent of enzyme inhibition was expressed as a percentage of remaining enzyme activity compared to the control. Each point represents the mean of four independent incubations and error bars represent the standard deviations of four independent determinations.

#### **4.2.2 Activity of norendoxifen and hydroxynorendoxifen to antagonize ER-responsive gene expression (in E2-stimulated breast cancer cells)**

To assess their antagonistic transcriptional activity in the presence of E2, norendoxifen (*E,Z*) and hydroxynorendoxifen (*E,Z*) were tested on the MCF-7 cells at a concentration of 1  $\mu$ M dissolved in minimum essential media (MEM) supplemented with 10% charcoal-stripped fetal bovine serum (FBS). As shown in Figure 12 (each result was expressed as mean  $\pm$  SD), the presence of 10 nM E2 was able to significantly increase the mRNA expression of progesterone receptor (PGR) gene compared to the control, which only contained 0.1% methanol (vehicle) in MEM supplemented with 10% charcoal-stripped FBS. PGR mRNA expression level with 10 nM E2 stimulation alone was considered as 100% PGR mRNA expression. Endoxifen was used as a positive control as it can antagonize PGR mRNA expression in the presence of 10 nM E2 to 10% expression level compared to 10 nM E2 stimulation alone, which is consistent with the previous published results<sup>40</sup>. Norendoxifen and hydroxynorendoxifen were able to antagonize PGR mRNA expression level to 32% and 14%, respectively. All the test compounds have shown statistically significant difference compared to the E2 alone stimulated control.





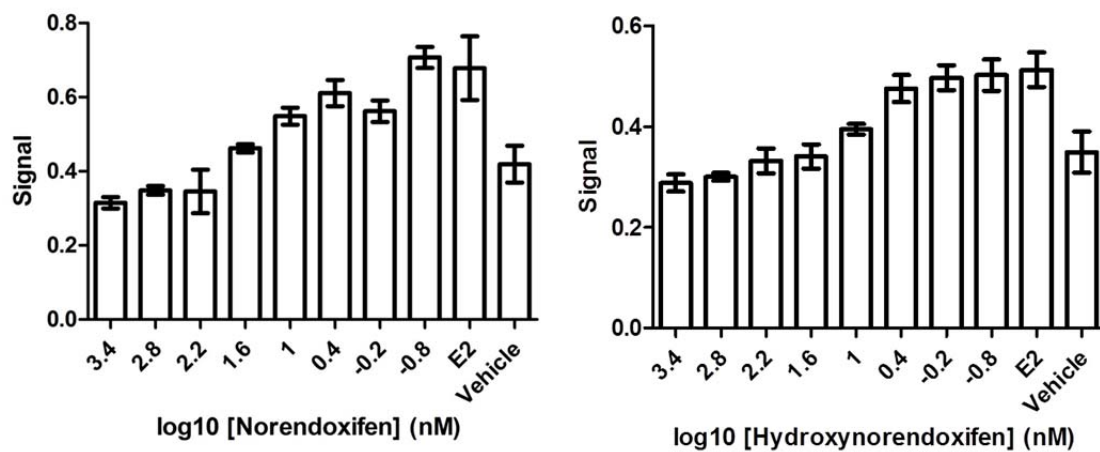
**Figure 12. Activity of norendoxifen and hydroxynorendoxifen to antagonize progesterone receptor (PGR) mRNA expression in MCF-7 cells**

MCF-7 cells were treated with 1000 nM norendoxifen and hydroxynorendoxifen in the presence of 10 nM 17 $\beta$ -estradiol (E2). PGR mRNA expression level with 10 nM E2 stimulation alone was considered as 100% PGR mRNA expression. Treatment with 1000 nM endoxifen was used as a positive control. Data represent mean  $\pm$  standard deviations from three independent determinations.

#### **4.2.3 Anti-proliferation activity of norendoxifen and hydroxynorendoxifen against breast cancer cells (stimulated by estradiol)**

The anti-proliferative effect of norendoxifen and hydroxynorendoxifen on human breast cancer cells (MCF-7 cells) was measured by MTT assay. As

shown in Figure 13, the presence of 1 nM 17 $\beta$ -estradiol (E2) was able to significantly increase the proliferation of MCF-7 cells compared to the controls, which only contained 0.1% methanol (vehicle) in MEM supplemented with 10% charcoal-stripped FBS. In the presence of norendoxifen or hydroxynorendoxifen, the growth of MCF-7 cells was inhibited, and the IG<sub>50</sub> (inhibiting growth by 50%) values of norendoxifen and hydroxynorendoxifen on MCF-7 cells were 25  $\pm$  12 nM and 9  $\pm$  3 nM, respectively.



**Figure 13. Anti-proliferative effects of norendoxifen (*E,Z*) and hydroxynorendoxifen (*E,Z*) on human breast cancer cells**

Human breast cancer cells (MCF-7 cells) viability was measured by MTT assay. To determine the anti-proliferative effect of norendoxifen (*E,Z*) and hydroxynorendoxifen (*E,Z*), MCF-7 cells were treated with different concentrations of norendoxifen (*E,Z*) and hydroxynorendoxifen (*E,Z*) in the presence of 1 nM 17 $\beta$ -estradiol (E2). Treatment of E2 alone and vehicle alone were used as controls, respectively. Data represent the mean  $\pm$  standard

deviations from three independent determinations, in each of which every concentration group contains six replicates with one assay.

### 4.3 Discussion

Results summary for chapter 4 (cellular aromatase inhibitory activity and ER antagonistic ability of norendoxifen and hydroxynorendoxifen):

- 1) Norendoxifen and hydroxynorendoxifen can inhibit cellular aromatase activity in MCF-7 cells with  $IC_{50}$  of 565 nM and 282 nM, respectively.
- 2) Norendoxifen and hydroxynorendoxifen can inhibit breast cancer cell proliferation with  $IG_{50}$  of 25 nM and 9 nM, respectively.
- 3) Norendoxifen and hydroxynorendoxifen can suppress progesterone receptor (PGR) gene mRNA expression level by reducing 68% and 86% PGR mRNA expression.

In this chapter, more work on cellular aromatase inhibition was done to confirm the aromatase inhibitory activity of norendoxifen and hydroxynorendoxifen. The results are consistent with our previous findings using human recombinant aromatase (CYP19) that hydroxynorendoxifen is a more potent AI than norendoxifen. Table 11 shows the  $IC_{50}$  values of norendoxifen and hydroxynorendoxifen against aromatase using two methods, and the ratios of  $IC_{50}$  values (cellular/recombinant aromatase inhibition) were also calculated.

**Table 11. IC<sub>50</sub> values of norendoxifen (*E,Z*) and hydroxynorendoxifen (*E,Z*) against cellular and recombinant aromatase activity**

|   | Norendoxifen | Hydroxynorendoxifen |
|---|--------------|---------------------|
| <b>Cellular aromatase inhibition (IC<sub>50</sub>, nM)</b>    | <b>565</b>   | <b>282</b>          |
| <b>Recombinant aromatase inhibition (IC<sub>50</sub>, nM)</b> | <b>131</b>   | <b>45</b>           |
| <b>Ratio (cellular/recombinant aromatase inhibition)</b>      | <b>4.3</b>   | <b>6.3</b>          |

The IC<sub>50</sub> values of norendoxifen and hydroxynorendoxifen against cellular aromatase are higher than those of recombinant aromatase. There might be three reasons leading to the difference: 1) these compounds have to cross the cell membrane to inhibit the aromatase activity; 2) efflux drug transporter on the cell membrane may pump these compounds out; 3) even though cofactor proteins have been incorporated into the microsomal incubations when testing hum recombinant aromatase inhibition, however, there may be some other cofactor proteins existing in the MCF-7 cells leading to binding of norendoxifen and hydroxynorendoxifen. Also, it is notable that the ratio of cellular/recombinant aromatase inhibition for hydroxynorendoxifen was higher than that for norendoxifen (6.3 fold for hydroxynorendoxifen versus 4.3 fold for norendoxifen), which may be caused by many reasons, such as cell membrane permeability due to the increased hydrophobic nature of hydroxynorendoxifen, compound stability, and protein binding.

According to previous studies (ATAC, IES, BIG-98), the third generation AIs (letrozole, anastrozole and exemestane) are superior to tamoxifen as drugs to reduce the recurrence of ER-positive breast cancer in post-menopausal women. However, their efficacy is compromised by toxicities that reduce quality of life and treatment adherence<sup>107,108</sup>. Obviously, new AIs with fewer side effects are needed to allow better treatment of ER-positive breast cancer in post-menopausal women. As we discussed previously, several studies have shown that the combination of a SERM/SERD agent and an AI have the potential to increase breast cancer tumor growth inhibition, reduce the risk of drug resistance, and alleviate side effects.

The results show that both norendoxifen and hydroxynorendoxifen possess dual SERM(D)/AI activity, and they can become attractive lead compounds for the development of dual SERM(D)/AI agents because of three unique features: 1) the potent inhibitory ability of norendoxifen and hydroxynorendoxifen are able to reduce estrogen biosynthesis in the breast to inhibit the tumor growth, while, its ER antagonistic activity make it possible to completely deplete the E2 effects; 2) since norendoxifen and hydroxynorendoxifen are metabolites of the most widely used SERM, tamoxifen, lots of patients have already been exposed to them supports a low-risk safety profile expected for norendoxifen and hydroxynorendoxifen; 3) since norendoxifen and hydroxynorendoxifen share the similar chemical structures with endoxifen and tamoxifen, they may have agonistic activity in some tissues, such as bone tissue. In this way, norendoxifen and hydroxynorendoxifen may

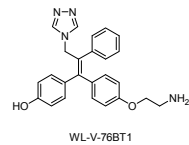
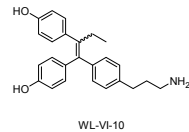
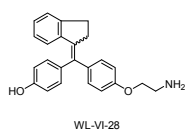
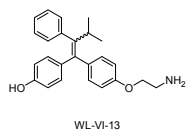
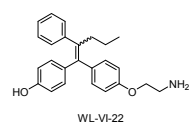
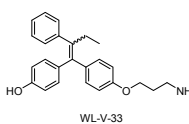
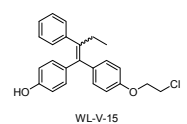
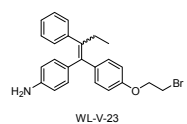
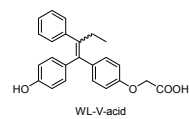
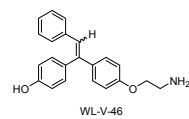
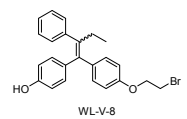
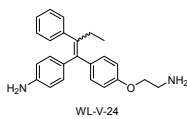
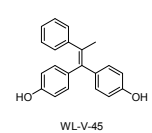
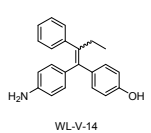
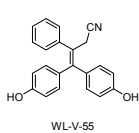
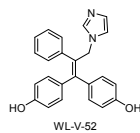
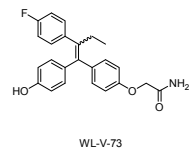
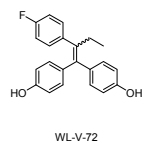
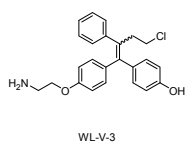
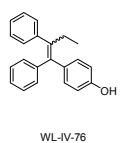
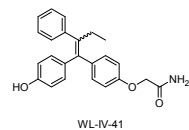
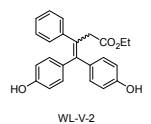
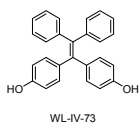
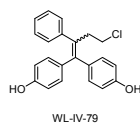
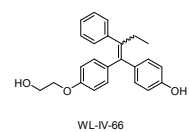
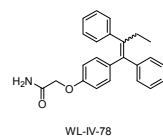
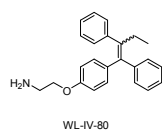
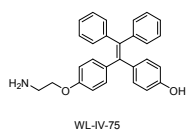
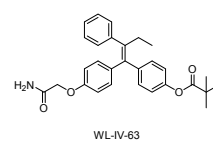
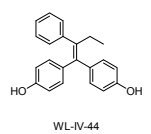
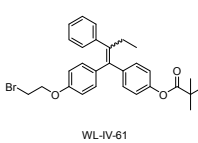
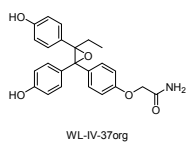
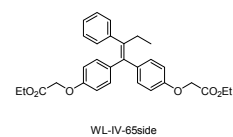
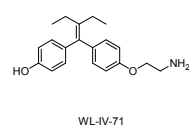
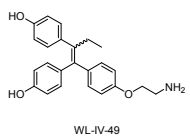
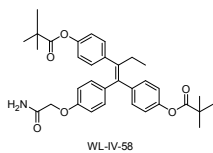
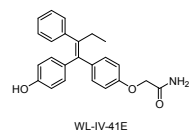
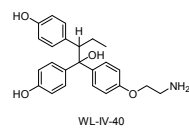
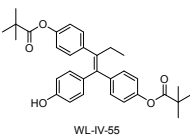
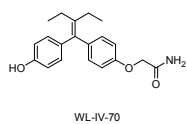
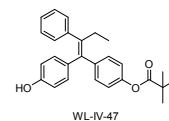
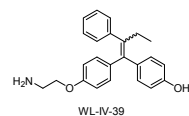
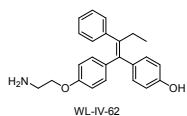
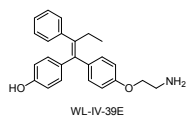
ameliorate some side effects in bone and other tissues caused by estrogen depletion.

The data reveal a complex metabolism of tamoxifen that may lead to some metabolites that are able to inhibit aromatase (CYP19) or act as a SERM or combine both of these activities. Also, the contribution of norendoxifen to the overall effects of tamoxifen remains unknown and its concentration in patients is not well defined. Studies have shown that endoxifen has low concentration in plasma, however, the tissue concentrations of endoxifen are higher, appearing to be 10 -100 times more<sup>105</sup>. The tissue concentration of norendoxifen may be higher, as demonstrated with endoxifen. Since norendoxifen has potent inhibitory ability against aromatase (CYP19), it may significantly increase the effects of tamoxifen *in vivo*. All of these possibilities deserve more study and are important for better understanding of tamoxifen function and novel drug development for the treatment of breast cancer.

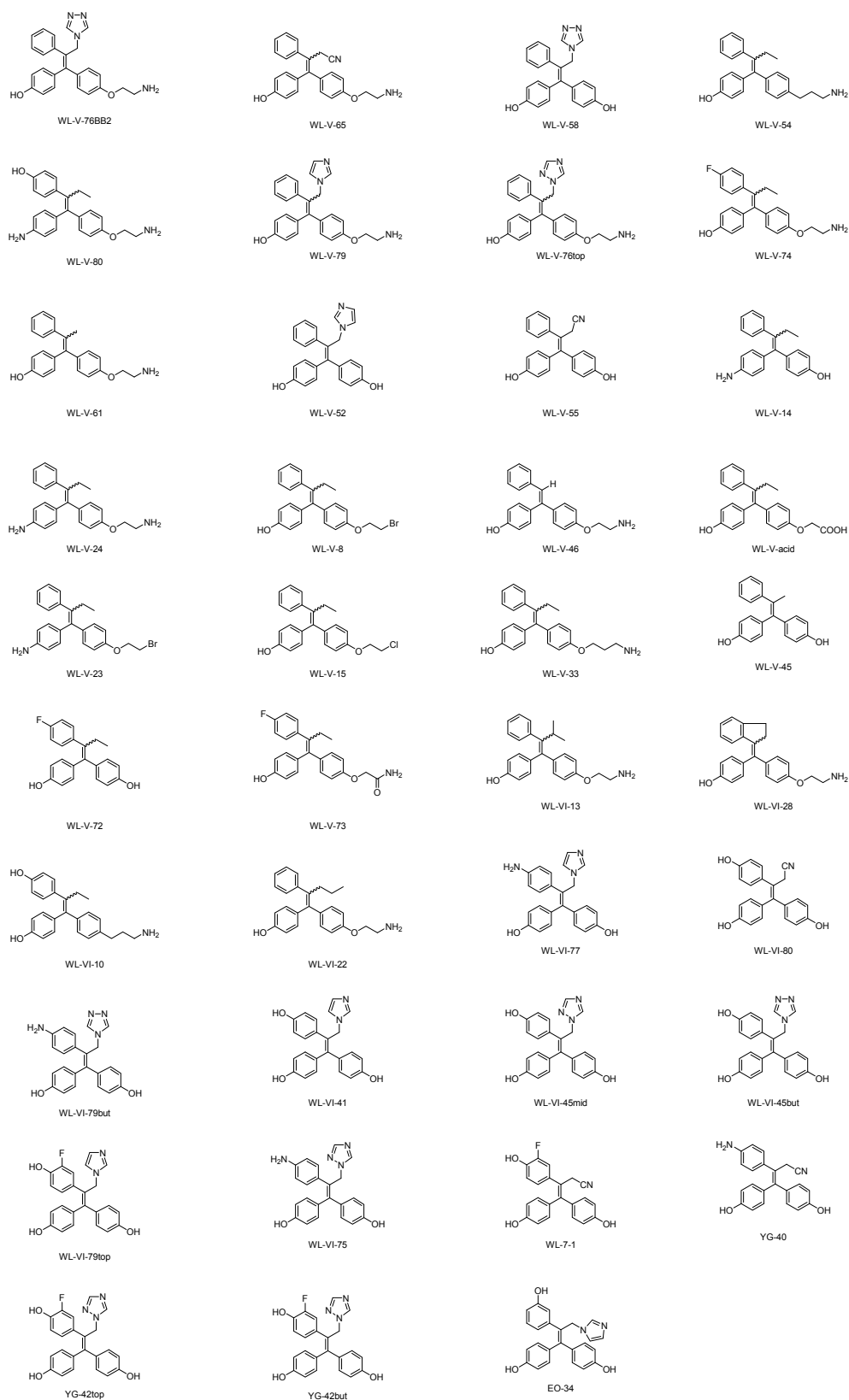
## **CHAPTER 5: CHARACTERIZATION OF NORENDOXIFEN ANALOGUES WITH DUAL AROMATASE INHIBITORY ABILITY AND ESTROGEN RECEPTOR ANTAGONISITIC ACTIVITY**

### **5.1 Introduction**

Previous studies by our group discovered that norendoxifen was a compound with dual aromatase inhibitory and ER antagonistic activity. To improve the efficacy and develop more lead compounds, 87 structurally related norendoxifen analogues were synthesized by Dr. Wei Lv, Dr. Liming Zhao, Dr. Elizaveta N O'Neill and Dr. Mark Cushman from our collaborating group at Purdue University. Figure 14 showed the chemical structures of the norendoxifen analogues.







**Figure 14. Chemical structures of norendoxifen analogues**

In this chapter, we will focus on **Aim 3** to characterize norendoxifen analogues with dual aromatase inhibitory activity and estrogen receptor antagonistic activity. Studies outlined in **Aim 3** test this hypothesis through the following specific sub-aims:

**Aim 3A.** To test the potency of aromatase inhibition by norendoxifen analogues *in vitro*.

**Aim 3B.** To characterize ER binding affinities of norendoxifen analogues *in vitro*.

**Aim 3C.** To test ER antagonistic activity of norendoxifen analogues *in vitro*.

## **5.2 Results**

### **5.2.1 Potency of aromatase inhibition and ER binding affinity by norendoxifen analogues *in vitro***

The IC<sub>50</sub> values of lead norendoxifen analogues against aromatase (CYP19) and the EC<sub>50</sub> values of lead norendoxifen analogues to ERs (ER- $\alpha$  and ER- $\beta$ ) are shown in Table 12 (values less than 500 nM were highlighted in red).

**Table 12. IC<sub>50</sub> values of lead norendoxifen analogues against aromatase (CYP19) and the EC<sub>50</sub> values of lead compounds to ERs (ER-α and ER-β)**

| Compounds  | IC <sub>50</sub> (nM) against Aromatase (CYP19) | EC <sub>50</sub> (nM) for ER-α binding affinity | EC <sub>50</sub> (nM) for ER-β binding affinity |
|------------|---|---|---|
| WL-IV-4    | 156 ± 1   | N.A.  | N.A.  |
| WL-IV-79   | 9194 ± 67                                       | 147 ± 4   | 20 ± 7  |
| WL-IV-73   | N.A.  | 311 ± 44  | 69 ± 7  |
| WL-V-52    | 4.8 ± 0.4                                       | 27 ± 5  | 41 ± 12   |
| WL-V-24    | 53 ± 2  | 274 ± 44  | 181 ± 82  |
| WL-V-33    | 1240 ± 176                                      | 35 ± 11   | 21 ± 6  |
| WL-V-3     | 143 ± 2   | 265 ± 7   | 52 ± 22   |
| WL-V-76BT1 | 715 ± 10  | 57 ± 11   | 48 ± 25   |
| WL-V-76BB2 | 699 ± 63  | 176 ± 54  | 58 ± 33   |
| WL-V-65    | 49 ± 12   | N.A.  | N.A.  |
| WL-V-58    | 137 ± 6   | N.A.  | N.A.  |
| WL-V-54    | 7339 ± 449                                      | 27 ± 15   | 13 ± 1  |
| WL-V-80    | 48 ± 8  | 144 ± 62  | 50 ± 9  |
| WL-V-79    | 17 ± 1  | N.A.  | N.A.  |
| WL-V-76top | 174 ± 10  | 290 ± 194                                       | 519 ± 345                                       |

|             |                |                 |                |
|-------------|----------------|-----------------|----------------|
| WL-V-74     | $1017 \pm 295$ | $88 \pm 6$      | $49 \pm 13$    |
| WL-V-61     | $337 \pm 13$   | $675 \pm 458$   | $28 \pm 19$    |
| WL-VI-22    | $261 \pm 40$   | $384 \pm 161$   | $36 \pm 9$     |
| WL-VI-13    | $328 \pm 53$   | N.A.            | N.A.           |
| WL-VI-28    | $493 \pm 29$   | $3176 \pm 243$  | $1346 \pm 550$ |
| WL-VI-77    | $5.2 \pm 0.4$  | $1826 \pm 910$  | $296 \pm 154$  |
| WL-VI-41    | $60 \pm 4$     | $98 \pm 42$     | $74 \pm 30$    |
| WL-VI-79but | $18 \pm 1$     | N.A.            | N.A.           |
| WL-VI-79top | $104 \pm 10$   | N.A.            | N.A.           |
| WL-VI-75    | $36 \pm 1$     | N.A.            | N.A.           |
| WL-7-1      | $94 \pm 4$     | $85 \pm 14$     | $56 \pm 18$    |
| ZHAO-I-20   | $221 \pm 42$   | $213 \pm 54$    | $486 \pm 239$  |
| ZHAO-I-28   | $62 \pm 8$     | $72 \pm 43$     | $70 \pm 5$     |
| ZHAO-I-39   | $230 \pm 11$   | $11036 \pm 828$ | $857 \pm 389$  |
| ZHAO-I-46   | $177 \pm 20$   | N.A.            | N.A.           |
| ZHAO-I-44   | $9 \pm 2$      | $1711 \pm 630$  | $1263 \pm 423$ |
| ZHAO-I-42   | $13 \pm 3$     | N.A.            | N.A.           |

|                  |                  |                  |                  |
|------------------|------------------|------------------|------------------|
| <b>ZHAO-I-50</b> | <b>287 ± 32</b>  | <b>451 ± 201</b> | <b>346 ± 115</b> |
| <b>ZHAO-I-51</b> | <b>645 ± 246</b> | <b>164 ± 97</b>  | <b>218 ± 102</b> |
| <b>EO-34</b>     | <b>60 ± 2</b>    | <b>N.A.</b>      | <b>N.A.</b>      |

Among the 87 norendoxifen analogues, 13 compounds (WL-V-52, WL-V-24, WL-V-3, WL-V-80, WL-V-76top, WL-V-61, WL-VI-22, WL-VI-77, WL-VI-41, WL-7-1, ZHAO-I-20, ZHAO-I-28 and ZHAO-I-50) had both potent aromatase inhibitory activity and high ERs binding affinity with IC<sub>50</sub> and EC<sub>50</sub> values less than 500 nM.

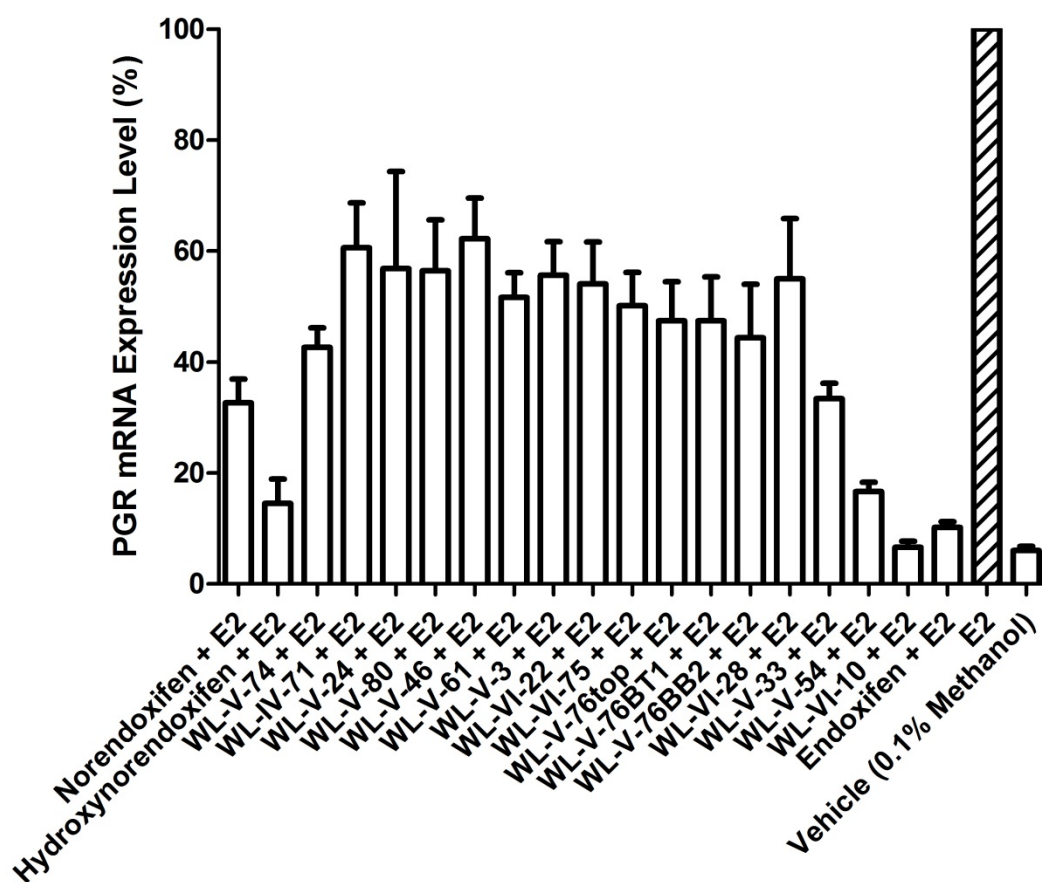
Among the 87 norendoxifen analogues, 14 compounds (WL-IV-4, WL-V-65, WL-V-58, WL-V-79, WL-VI-13, WL-VI-28, WL-VI-79but, WL-VI-79top, WL-VI-75, ZHAO-I-39, ZHAO-I-46, ZHAO-I-44, ZHAO-I-42 and EO-34) had only potent aromatase inhibitory activity with IC<sub>50</sub> values less than 500 nM.

Among the 87 norendoxifen analogues, 8 compounds (WL-IV-79, WL-IV-73, WL-V-33, WL-V-76BT1, WL-V-76BB2, WL-V-54, WL-V-74 and ZHAO-I-51) had only high ERs binding affinity with EC<sub>50</sub> values less than 500 nM.

### **5.2.2 ER antagonistic activity of norendoxifen analogues *in vitro***

To assess the antagonistic transcriptional activity of norendoxifen analogues in the presence of 17β-estradiol (E2), 18 norendoxifen analogues were tested on MCF-7 cells at a concentration of 1 μM dissolved in minimum essential media (MEM) supplemented with 10% charcoal-stripped fetal bovine

serum (FBS). The reason for choosing these 18 compounds is that they have relatively high ER binding affinities for ER- $\alpha$  or ER- $\beta$  or both. As shown in Figure 15 (each result was expressed as mean  $\pm$  SD), the presence of 10 nM E2 was able to significantly increase the mRNA expression of progesterone receptor (PGR) gene compared to the blanks, which only contained 0.1% methanol (vehicle) in MEM supplemented with 10% charcoal-stripped FBS. PGR mRNA expression level with 10 nM E2 stimulation alone was considered as 100% PGR mRNA expression. Endoxifen was used as a positive control as it can antagonize the PGR mRNA expression in the presence of 10 nM E2 to 10% expression level compared to 10 nM E2 stimulation alone, which is consistent with the previous published results<sup>40</sup>. Among the 18 test compounds, norendoxifen, hydroxynorendoxifen, WL-V-74, WL-V-76top, WL-V-76BT1, WL-V-76BB2, WL-V-33, WL-V-54 and WL-VI-10 were able to antagonize the PGR mRNA expression level to 32%, 14%, 42%, 47%, 47%, 44%, 33%, 16% and 6%, respectively. All the test compounds have shown statistically significant differences as compared to the E2 alone stimulated control.



**Figure 15. Activity of lead norendoxifen analogues to antagonize progesterone receptor (PGR) mRNA expression in MCF-7 cells**

MCF-7 cells were treated with 1000 nM lead norendoxifen analogues in the presence of 10 nM 17 $\beta$ -estradiol (E2). PGR mRNA expression level with 10 nM E2 stimulation alone was considered as 100% PGR mRNA expression.

Treatment with 1000 nM endoxifen was used as positive control. Data represent the mean  $\pm$  standard deviations from 3 independent determinations.

### 5.3 Discussion

Results summary for chapter 5 (characterization of norendoxifen analogues with dual aromatase inhibitory ability and estrogen receptor antagonistic activity):

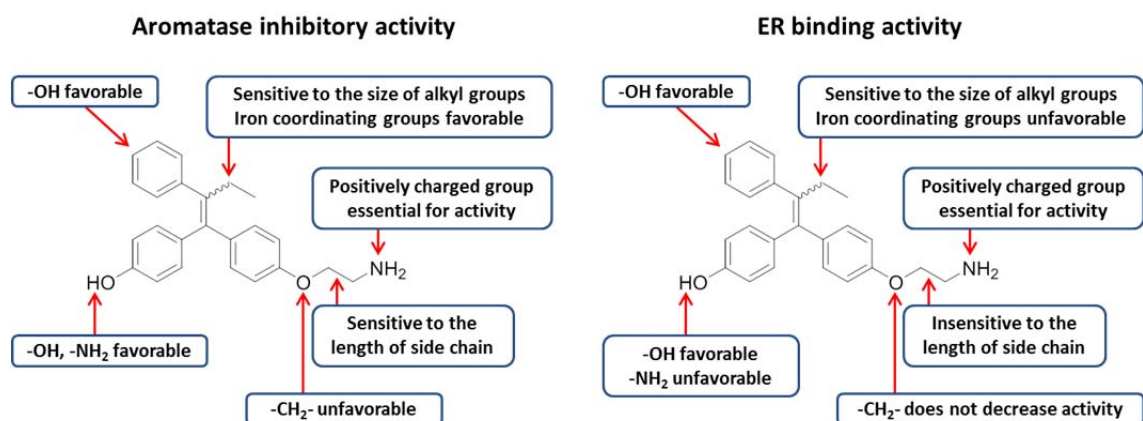
- 1) Among the 87 norendoxifen analogues, 13 compounds had both potent aromatase inhibitory activity and high ERs binding affinity with  $IC_{50}$  (aromatase inhibition) or  $EC_{50}$  (binding affinity) values less than 500 nM.
- 2) Among the 87 norendoxifen analogues, 14 compounds had only potent aromatase inhibitory activity with  $IC_{50}$  values less than 500 nM.
- 3) Among the 87 norendoxifen analogues, 8 compounds had only high ERs binding affinity with  $EC_{50}$  values less than 500 nM.

As we know, potent and selective AIs with limited side effects are needed to improve the treatment of hormone receptor-positive breast cancer in post-menopausal women. Norendoxifen has the potential to be a therapeutically useful AI with fewer side effects, and in the meantime, it provides a new lead compound for the rational design of a series of novel compounds with dual aromatase inhibitory activity and ER modulatory activity.

A series of structurally related norendoxifen analogues were tested for their aromatase inhibitory activity and binding affinity to ERs. Moreover, the antagonistic activity of norendoxifen analogues with relatively high ERs binding affinity was tested against estrogen-responsive genes. Among these norendoxifen analogues: some compounds had both potent aromatase inhibitory activity and high ERs binding affinity; some compounds had only potent



aromatase inhibitory activity; some compounds had only potent aromatase inhibitory activity. As shown in Figure 16 and Table 13, the structure-activity relationships were discussed, and this would support the development of lead optimization. For lead compounds with potent aromatase inhibitory activity and high ERs binding affinity, their chemical structures have common features: 1) -OH group in 4'-position; 2) -OH group in 4''-position; 3) positively charged group at the end of side chain at 4-position. For lead compounds with only potent aromatase inhibitory activity, their chemical structures may have -NH<sub>2</sub> group in 4'-position, iron coordinating groups in 9-position and may not have -CH<sub>2</sub>- group in 4-position. Also their chemical structures may be sensitive to the size of alkyl groups in 9-position and sensitive to the length of side chain at 4-position. For lead compounds with only high binding affinity to ERs, their chemical structures may not have an -NH<sub>2</sub> group at 4'-position or iron coordinating groups at 9-position.



**Figure 16. Summary of structure-activity relationships**

**Table 13. Summary of structure-activity relationships**

| <b>Activity</b>   | <b>Chemical groups and their positions</b>  |
|---|---|
| <b>Favorable for both aromatase inhibitory activity and ER binding affinity</b> | <b>-OH group at 4'-position, -OH group at 4''-position, positively charged group at end of side chain of 4-position</b> |
| <b>Favorable for aromatase inhibitory activity only</b>                         | <b>-NH<sub>2</sub> group at 4'-position, iron coordinating groups at 9-position</b>                                     |
| <b>Favorable for ERs binding affinity only</b>                                  | <b>Insensitive to the length of side chain at 4-position</b>  |

According to our findings of the structure-activity relationships, the predicted chemical structure of a strong SERM/AI agent is that of hydroxynorendoxifen.

## CHAPTER 6: SUMMARY AND CONCLUSIONS

Results summary for this dissertation:

- 1) Both norendoxifen and hydroxynorendoxifen are tamoxifen metabolites, and they are potent and relatively selective AIs with  $K_i$  values of 70 nM and 20 nM via a competitive mechanism *in vitro*, respectively.
- 2) Norendoxifen and hydroxynorendoxifen have high binding affinity against ER- $\alpha$  and ER- $\beta$ , with  $EC_{50}$  values less than 35 nM.
- 3) Norendoxifen and hydroxynorendoxifen can inhibit breast cancer cell proliferation with high potency, with  $IG_{50}$  values of 25 nM and 9 nM, respectively.
- 4) Norendoxifen and hydroxynorendoxifen can suppress progesterone receptor (PGR) gene mRNA expression level by reducing 68% and 86% PGR mRNA expression.
- 5) A series of norendoxifen analogues were developed as lead compounds for the development of dual AI/SERM(D) agents.

Results from this dissertation will contribute to three aspects: 1) the identification of hydroxynorendoxifen as a tamoxifen metabolite illustrated a more comprehensive metabolism profile of tamoxifen; 2) the data suggest norendoxifen and hydroxynorendoxifen possess dual aromatase inhibitory and ER antagonistic activity; 3) a series of norendoxifen analogues were developed as lead compounds for the development of dual AI/SERM(D) agents.

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affect outcome in breast cancer patients treated with tamoxifen  
monotherapy. *Breast Cancer Res. Treat.* **125**, 279-287,  
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## CURRICULUM VITAE

Jin-Zhong Liu

### EDUCATION

**Ph.D. in Biochemistry and Molecular Biology** Sep 2011 – Nov 2015

Department of Biochemistry and Molecular Biology, Indiana University,  
Indianapolis, IN

*Mentor: David A. Flockhart, M.D., Ph.D.*

**B.S. in Material Chemistry** Sep 2007 – Jul 2011

College of Chemistry, Nankai University, Tianjin, China

### WORK EXPERIENCE

**Division of Clinical Pharmacology, Indiana University School of Medicine,  
Indianapolis, IN**

**Research assistant** with Dr. David Flockhart Jul 2012 – Aug 2015

- Developed a population PK/PD model of tamoxifen and its active metabolites associated with tamoxifen-induced side effect hot flushes using clinical trial data from breast cancer patients
- Performed covariate modeling to test whether CYP2D6 genetic polymorphisms affect the pharmacokinetics of tamoxifen active metabolites 4-hydroxytamoxifen and endoxifen
- Characterized the inhibition of cytochrome P450 enzymes by *E*-, *Z*-isomers of norendoxifen

- Identified the analogs of norendoxifen as aromatase inhibitors and estrogen receptor modulators
- Identified and quantified a novel tamoxifen metabolite, hydroxynorendoxifen, serves as a potent and selective aromatase inhibitor for treatment of hormone receptor-positive breast cancer
- Characterized norendoxifen and hydroxynorendoxifen as antagonists for estrogen receptors (ERs) in breast cancer cells

## **PUBLICATIONS**

1. **Jin-Zhong Liu**, Peter J. Flockhart, De-Shun Lu, Wei Lv, Wen-Jie Jessie Lu, Xu Han, Mark Cushman and David A. Flockhart (2013) "*Inhibition of Cytochrome P450 Enzymes by E-, Z-Isomers of Norendoxifen*", Drug Metabolism and Disposition 41: 1715-1720.
2. Wei Lv, **Jin-Zhong Liu**, Todd Skaar, David A. Flockhart and Mark Cushman (2015) "*Design and Synthesis of Norendoxifen Analogues with Dual Aromatase Inhibitory and Estrogen Receptor Modulatory Activities*", Journal of Medicinal Chemistry 58: 2623–2648.
3. Wei Lv, **Jin-Zhong Liu**, De-Shun Lu, David A. Flockhart and Mark Cushman (2013) "*Synthesis of Mixed (E,Z)-, (E)-, and (Z)-Norendoxifen with Dual Aromatase Inhibitory and Estrogen Receptor Modulatory Activities*", Journal of Medicinal Chemistry 56: 4611-4618.

4. Qin Ouyang, Kai-Qi Yan, Yi-Zhou Zhu, Cai-Hui Zhang, **Jin-Zhong Liu**, Chen Chen, and Jian-Yu Zheng (2012) "*An Efficient PIFA-Mediated Synthesis of a Directly Linked Zinc Chlorin Dimer via Regioselective Oxidative Coupling*", Organic Letters 14: 2746-2749.

## **ABSTRACTS**

1. **Jin-Zhong Liu**, Brian Corrigan, Kaori Ito, Kevin Sweeney, Jing Liu, David A. Flockhart and Timothy Nicholas, "*A Structural Model Characterizing Placebo Effect for the Children's Depression Rating Scale (CDRS) in a Pediatric Major Depressive Disorder Population*", 2015 Annual Meeting of ASCPT, New Orleans, LA.
2. **Jin-Zhong Liu**, Brian Corrigan, Timothy Nicholas, Kaori Ito and David A. Flockhart, "*MARS (Meta-Analysis using R Shiny): A Browser Based Meta-Analysis Modeling Visualization Application*", 2015 Annual Meeting of ASCPT, New Orleans, LA.
3. **Jin-Zhong Liu**, Timothy Nicholas, Jing Liu, Kaori Ito, Tina Checchio, Sima Ahadieh, David A. Flockhart and Brian Corrigan, "*SMARTR (Shiny Meta-Analysis Research Tool using R): A Browser Based Meta-Analysis Visualization tool using R Shiny*", 2014 Annual Meeting of ACoP, Las Vegas, NA.
4. **Jin-Zhong Liu**, Timothy Nicholas and Brian Corrigan, "*Application of Web-based Interactive R-based Models using Shiny*", 2014 Annual Meeting of

ACoP, Las Vegas, NV.

5. **Jin-Zhong Liu**, De-shun Lu, Jessica Bo Li Lu, Wei Lv, Zeruesenay Desta, Mark Cushman and David A. Flockhart, “*Hydroxynorendoxifen, a Tamoxifen Metabolite, Possesses Dual Aromatase Inhibitory and Estrogen receptor Modulatory Activities*”, 2014 Annual Meeting of ASCPT, Atlanta, GA.
6. **Jin-Zhong Liu**, Peter J. Flockhart, De-Shun Lu, Wei Lv, Mark Cushman and David A. Flockhart, “*Inhibition of Cytochrome P450 Enzymes by E-, Z-Isomers of Norendoxifen*”, 2013 Annual Meeting of ASCPT, Indianapolis, IN.
7. **Jin-Zhong Liu**, Wen-Jie Jessie Lu, Peter J. Flockhart, De-Shun Lu, Wei Lv, Xu Han, Mark Cushman and David A. Flockhart, “*Norendoxifen, an Active Metabolite of Tamoxifen, is a Potent and Selective Aromatase Inhibitor*”, 2013 Annual Meeting of ASCPT, Indianapolis, IN.
8. **Jin-Zhong Liu**, Wei Lv, De-Shun Lu, Mark Cushman and David A. Flockhart, “*Analogues of Norendoxifen Serve as Aromatase Inhibitors and Selective Estrogen Receptor Modulators*”, 2013 Annual Meeting of ASCPT, Indianapolis, IN.

### **AWARDS & HONORS**

- 2015 ASCPT David J. Goldstein Trainee Award
- 2015 ASCPT (American Society of Clinical Pharmacology & Therapeutics) Presidential Trainee Award

- 2014 ISoP (International Society of Pharmacometrics) Outstanding Trainee Award